Modulation of Cargo Transport and Sorting through Endosome Motility and Positioning

A dissertation submitted to the
Technical University of Dresden

For the degree of
Doctor rerum naturalium
In Biology

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I. Declaration

I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign association board.

The thesis work was conducted from 01.07.2002 to 30.5.2005 under the supervision of Prof. Dr. Marino Zerial at the Max-Planck Institute of Molecular Cell Biology and Genetics in Dresden, Germany.

II. Acknowledgements

I would like to express my gratitude to Marino Zerial and his group for providing an exciting research environment and an inspiring atmosphere where I could freely expand my scientific thinking. Furthermore, I wish to thank my Ph.D. thesis committee Prof. Dr. Tony Hyman, Prof. Dr. Gerold Barth and Prof. Dr. Zerial for their time and guidance. With respect to the research on KIF16B, I am particulary grateful to Prof. Dr. Harald Stenmark and Alicia Cabezas for their great support and helpful discussions. Furthermore, I would like to express my gratitude to Fedor Severin who greatly contributed to the work on molecular motors with his expertise in *in vitro* motility studies. Last but not least, I would like to thank Bianca Habermann for her expert support with bioinformatics problems and Anja Runge and David Gillooly for their collaborative efforts. I would also like to acknowledge Julia Keller who recently joined the *C.elegans* screening project and who already shares with me all the joy and all the hassle which goes along with this mission. Furthermore, I would like to thank all musicians in the various bands that I have played with over the years for their friendship and providing an excellent means of counter balancing intellectual stunts in science with heavy metal, alternative rock and jazz. I also thank my parents for their love and care and last but not least all my love to Maike Gravert who has motivated and supported me throughout these years and who gave me a wonderful introduction into how difficult it is to play saxophone or to do horse-back riding…

III. ABSTRACT

During the past years, substantial progress has been made towards the elucidation of the basic mechanisms underlying transport in the secretory and endocytic pathways. Utilizing various systems such as cell-based assays but also multicellular organisms such as *Drosophila melanogaster* and *C.elegans*, for example, the endocytic system has been shown to consist of a network of biochemically and morphologically distinct organelles that carry out specialized tasks in the uptake, recycling and catabolism of growth factors and nutrients, serving a plethora of key biological functions (Mellman, 1996). Different classes of endosomes were found to exhibit a characteristic intracellular steady state distribution. Whereas early endosomes are dispersed throughout the cytoplasm, recycling endosomes, late endosomes and lysosomes are typically situated in close proximity to the nucleus. This distribution pattern observed at steady state results from a dynamic interaction of endosomes with the actin and the microtubule cytoskeleton. It remains unclear, however, which microtubule-based motors besides Dynein control the intracellular distribution and motility of early endosomes and how their function is integrated with the sorting and transport of cargo.

On early endosomes, the small GTPase Rab5 coordinates the activity of several effector proteins that cooperatively regulate endocytic trafficking (Zerial and McBride, 2001). A key effector is the phosphatidylinositol-3-OH kinase (PI3-K) hVPS34/p150 that generates the phospholipids phosphatidylinositol(3)-phosphate (PI(3)P) in proximity to Rab5. The finding that the concerted activity of Rab5 and hVPS34 is also required for bidirectional, microtubule-based, endosome motility *in vitro* (Nielsen et al., 1999) raises the possibility that on early endosomes, the phospholipid PI(3)P may directly recruit an adaptor of a motor or, in the simplest case, a motor itself.

The first part of this thesis research outlines the search for such motor. I describe the identification of KIF16B which functions as a novel endocytic motor protein. This molecular motor, a kinesin-3, transports early endosomes to the plus end of microtubules, in a process regulated by the small GTPase Rab5 and its effector, the phosphatidylinositol-3-OH kinase hVPS34. In vivo, KIF16B overexpression relocated

early endosomes to the cell periphery and inhibited transport to the degradative pathway. Conversely, expression of dominant-negative mutants or ablation of KIF16B by RNAi caused the clustering of early endosomes to the peri-nuclear region, delayed receptor recycling to the plasma membrane and accelerated degradation. These results suggest that KIF16B, by regulating the plus end motility of early endosomes, modulates the intracellular localization of early endosomes and the balance between receptor recycling and degradation. In displaying Rab5 and PI(3)P-containing cargo selectivity, a remarkable property of KIF16B is that it is subjected to the same regulatory principles governing the membrane tethering and fusion machinery (Zerial and McBride, 2001). Since KIF16B can modulate growth factor degradation, we propose that this motor could have also important implications for signaling. Importantly, KIF16B has provided novel insight into how intracellular localization of endosomes governs the transport activity of these organelles.

The second part of this thesis describes a more long-term project aimed at gaining insights into the next level of understanding: How the spatial distribution of organelles is linked to their function in an experimental system which features cellular polarity, for example, a tissue or organ. The suitability of *C. elegans* as a model system to identify genes functioning in endocytosis has been demonstrated by previous genetic screens (Grant and Hirsh 1999; Fares and Greenwald, 2001). This encouraged me to complement their efforts by devising a visual screening method to identify genes important for epithelial cell polarity. In particular, my work provides the foundation for future work to address the following unresolved questions in the field of endocytic trafficking:

- Why do apical endosomes maintain their polarized localization and which components regulate transport between such endosomes and the cell surface?
- How does a polarized cell regulate the size, shape, subcellular distribution as well as the number of transport vesicles at steady state?
- How do the endocytic recycling pathways contribute to the generation and maintenance of cell polarity?

Offering excellent morphological resolution and polarization, the nematode intestine represents a good system to study the apical sorting of a transmembrane marker. The steady state localization of such a marker is likely the result of a dynamic process that depends on biosynthetic trafficking to the apical surface, apical endocytosis and recycling occurring through apical recycling endosomes. Therefore, mis-sorting of this marker upon RNA-mediated interference will be indicative of a failure in one of the aforementioned processes. Furthermore, since it is still largely unclear why apical endosomes maintain their polarized localization, this screen will also monitor the morphology of this endocytic compartment using a second marker. A search for suitable marker proteins yielded several marker candidates, including Rab11 (as a marker for the recycling endosome) and Opt-2 (which is sorted apically) which were chosen for the primary screen. Utilizing the Ahringer's feeding library (Kamath and Ahringer, 2003) liquid cultured, synchronized worms are raised and silenced in a fully automated process. Following image acquisition based on an automated confocal microscope, data can be analyzed using custom-built software allowing objective phenotypic analysis. A primary small-scale proof-of-principe screen has been conducted for each marker and phenotypes were found. The successful establishment of the proof-of-principle marks the current state-of-the-art of this ambitious screening project. With high-throughput screening within reach, all 16753 C.elegans genes contained in the Ahringer library will be screened, secondary assays will be applied and all data will be clustered according to functionality in the near future. Ultimately, this screen will broaden our insights into what genes are implicated in apical transport and recycling on one hand and what genes are involved in the biogenesis and morphological maintenance of recycling endosomes in polarized epithelial cells on the other hand.

IV. CONTENTS

V. INTRODUCTION

V.I. Preface

The following paragraphs will sketch an overview over the cellular processes involved in endocytosis and sorting of internalized cargo. Furthermore, key molecular and structural components necessary for the canonical, receptor-mediated and clathrin dependent endocytic traffic pathways shall be presented here. In this context, the role of phospholipids, Rab proteins, the cytoskeleton as well as molecular motors will be highlighted. Due to the overwhelming complexity of this area of research, more specialized aspects of endocytosis such as neuronal endocytosis (Sudhof, 2004), endocytosis in development (Dudu et al., 2004) and non-clathrin dependent endocytosis pathways (Laude and Prior, 2004) will not be covered in detail here. Instead, where appropriate, references of review articles will be given which provide a more detailed coverage of those special topics.

V. II. Overview over endocytic pathways

Endocytosis is a means by which Eukaryotic cells take up nutrients and communicate with their environment. Next to various ion-channels and transport systems for small molecules, endocytosis represents an energy-dependent mechanism which enables the cell to internalize proteins, lipids and even particles and entire pathogenic organisms. Figure 1 gives an overview of the more common modes of internalization and illustrates subsequent intracellular traffic routes. The various entry routes include receptor mediated, clathrin dependent endocytosis (1) and non-clathrin dependent mechanisms such as phagocytosis (4), macropinocytosis (3) and uptake into small (ca. 60nm) invaginations called caveolae (2). The sorting of cargo into caveolae is thought to involve membrane domains ("rafts") of liquid ordered phase, rich in cholesterol- and glycosphingolipids (in yellow). Furthermore, there is evidence that, following a dynamin-dependent fission step, caveosomes (CAV) can exchange cargo with early endosomes

(Pelkmans et al., 2004). It is clear that viruses and toxins frequently enter the cell by hijacking the caveosome pathway, whereas it is not understood what physiological function this pathway has in a healthy tissue. Particles larger than 300nm are too big for clathrin lattices (see below) and are phagocytosed (see (Aderem, 2002) for review) to be ultimately degraded in lysosomes (LYS) while fluid phase droplets of extracellular medium can be internalized via macropinocytosis (3) (Amyere et al., 2002). Macropinosomes (MP) frequently form upon growth factor-induced membrane ruffling - a process that depends on actin polymerization. It should be mentioned here that additional routes of uptake such as non-clathrin, non-caveolae mediated endocytosis have been described but have not been studied as extensively at a molecular level and are omitted in the cartoon for clarity.

The canonical, receptor mediated and clathrin dependent endocytic process commences, when a ligand such as a growth factor, for example, binds its target receptor at the plasma membrane. The receptor can subsequently change conformation (induced fit) to trigger the assembly of a proteinaceous coat underneath the plasma membrane. Alternatively, receptors may also be endocytosed constitutively, as is the case for transferrin receptors, for example. In this case, endocytosis occurs independently of receptor bound ligand. Common concepts applied during coat formation and disassembly and the types of coats known to date shall be introduced later. In the case of internalizing receptors from the plasma membrane, clathrin triskelia (see below) combine to form a pit coated by a clathrin lattice. It is still under debate what components are necessary and sufficient to provide the forces necessary to induce membrane invagination and bud formation. After fission of the clathrin coated vesicle (CCV) from the plasma membrane, the coat is disassembled and the vesicle fuses with the early endosome (EE) also referred to as *the compartment of uncoupling receptor from ligand* (CURL) (Geuze et al., 1983). The early endosome has primarily the task of sorting cargo which should recycle back to the plasma membrane (principles underlying sorting processes in the endocytic network shall be discussed later) from cargo that is destined to be targeted to the Golgi or to be degraded in lysosomes. Recycling of cargo back to the cell's surface can either occur with fast kinetics ($t_{1/2} \sim 2$min) directly from the early endosome or it can occur over

recycling endosomes (RE) with slower kinetics ($t_{1/2}$ ~ 12 -14min) (Maxfield and McGraw, 2004).

Cargo sorting towards late endosomes (LE) occurs at the level of the early endosome. Cargo destined to be degraded is sorted into internal vesicles forming multi-vesicular bodies (MVB) transport intermediates also called endosomal carrier vesicles (ECVs). Once they have detached, ECVs/MVBs cannot fuse back with early endosomes and are transported along microtubules to late endosomes. Late endosomes, which are morphologically and biochemically distinct from ECVs represent the next sorting platform along the endocytic route. Here, cargo can be sorted to the Golgi, the plasma membrane or to lysosomes. Lysosomes (LYS), with their acidic pH and hydrolytic environment, represent the terminal destination for some endocytosed cargo.

V. III. Compartment identity

One of the major challenges in studying membrane traffic in general and endocytosis in particular is that all compartments involved are very dynamic in space and time. These compartments are not stable entities in a sense that frequently one compartment converts into another one – a process also known as maturation. What then is the best way of classifying endocytic organelles? The following characteristics have been used to assign identity to various endocytic organelles:

- pH
- function (e.g. cargo sorting)
- ultra-structure
- lipid content
- protein/enzyme markers

V. III. 1. Functional, biochemical and ultra-structural features of endocytic compartments

Various endocytic compartments differ from each other by their internal pH. Endocytosed cargo also frequently relies on this proton gradient for efficient sorting. For

example, viruses that enter the cell via endocytosis escape their degradation in lysosomes (pH5.0) by a pH sensitive fusion process of their coats with the membranes of either early or late endosomes. While the Semliki Forest virus, as an example, triggers this fusion event in early endosomes at a pH of around 6.2, the influenza virus requires a more acidic pH of 5.4 which is typically found in late endosomes (Smith and Helenius, 2004). Recycling endosomes were shown to have already a more neutral pH of 6.5 (Yamashiro et al., 1984). As mentioned earlier, early endosomes can differentially sort cargo into either the recycling or the degradative branch of endocytic transport. One possible way of characterizing endosomes therefore is based on cargo sorting and pH. It should be emphasized, however, that most sorting process never reach absolute efficiency. Clearly, additional features are required to assign identity to these traffic compartments. Next to ultra-structural features, such as the multivesicular structure of late endosomes as well as the multilamellar morphology of lysosomes in APCs (Murk et al., 2004), one can also classify endosomes based on lipid content of their membranes but also the presence of various protein markers such as, for example, small GTPases of the *ras* superfamily which will be covered in the following section.

V. III. 1. Rab GTPases

The familiy of Rab (isolated for the first time from *ra*t *b*rain) GTPases encompassing about 63 proteins within the human genome (Bock et al., 2001) regulate a plethora of membrane traffic and signaling events (Miaczynska et al., 2004b; Zerial and McBride, 2001). These regulatory proteins localize to distinct intracellular membrane compartments (see figure 1 for an overview of characterized Rab proteins). For example, Rab5 primarily localizes to the canonical early endosome, but also to the plasma membrane and clathrin coated vesicles (Chavrier et al., 1990; Horiuchi et al., 1995). Rab5 therefore is restricted to function early in the endocytic pathway. In contrast, many rab proteins such as Rab4, Rab7, Rab9 and Rab11 function downstream of Rab5. Both Rab4 and Rab11 GTPases are necessary to recycle cargo back to the plasma membrane. Both Rab7 and Rab9 localize to late endosomes where Rab9 mediates transport to the *trans*-Golgi network (TGN) (Lombardi et al., 1993). Upon endocytosis, cargo molecules such

as transferrin have been shown to first enter Rab5 positive structures and later traverse Rab4 and subsequently Rab11 compartments when recycling back to the surface (Sonnichsen et al., 2000).

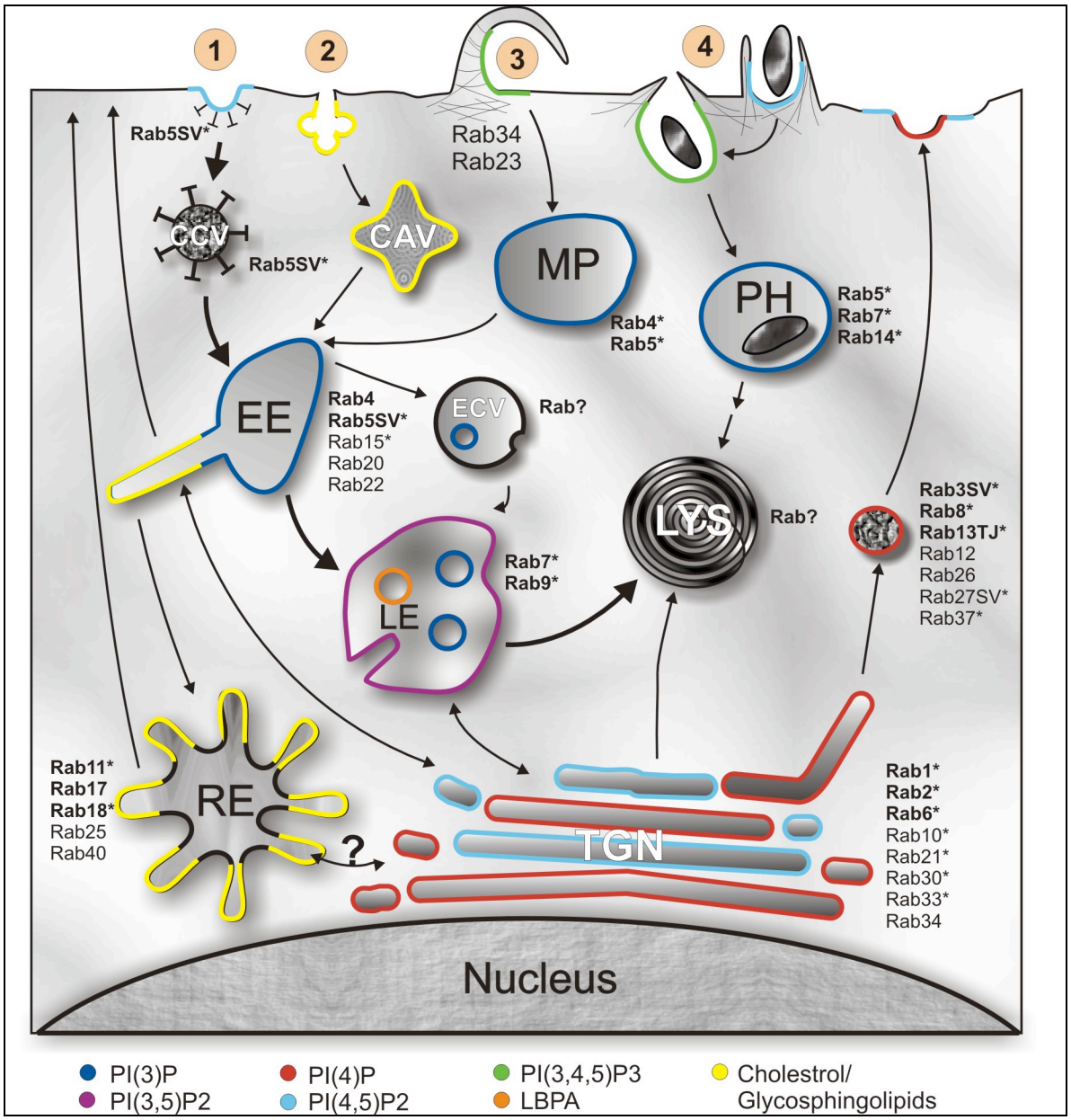


Figure 1: Steady state localization of phospholipids and Rab GTPases in the endo- and exocytic pathways. *Main entry-routes into the cell include receptor mediated endocytosis (1), the caveosome pathway (2), macropinocytosis (3) and phagocytosis (4). During endocytosis, PI(4,5)P2 is required for the formation and internalization of clathrin coated vesicles (CCVs). PI(3)P is present on phagosomes (PH), macropinosomes (MP),*

early endosomes (EE) and the internal vesicles of multivesicular bodies (MVB) and late endosomes (LE). Caveolae, caveosomes (CAV), and recycling endosomes (RE) are enriched with lipids forming liquid ordered phase 'rafts'. No particular enrichement of phosphatidylinositides has been observed on endosomal carrier vesicles (ECVs) or on lysosomes (LYS). The generation of PI(4,5)P2 and PI(4)P on the trans-golgi-network (TGN) is critical for exocytosis. The steady state localization of Rab GTPases as well as trafficking routes interconnecting the various organelles are indicated. Since this thesis includes work done in the C.elegans system, Rab proteins which are conserved in this nematode (Bock et al., 2001) have been marked by an asterisks (*).

Rab proteins continuously oscillate between membrane-bound and cytosolic localization (see Figure 2). This cycle is tightly regulated by various accessory factors and directly depends on a second cycle in which the Rab protein switches back and forth between a GDP and a GTP-bound form (see below). The monomeric Rab proteins insert into membranes using their geranylgeranyl lipid modification at their hypervariable C-terminus. In cytosol, the Rab-specific chaperone GDI (GDP-dissociation-inhibitor (Seabra and Wasmeier, 2004)) binds the hydrophobic lipid moiety of GDP-bound Rab molecules to prevent their aggregation and degradation (see Figure 2). It is not fully understood what controls the spatial restriction of Rab GTPases to certain compartments. In classical chimera experiments (Chavrier et al., 1991) it could be shown that the hypervariable domain can confer target specifity upon a Rab protein. For example, Rab5 harbouring the c-terminal sequence of Rab7 would relocalize to Rab7 positive, late endosomes. For early endosomes, the GDI-displacement factor (GDF) Pra-1 was postulated to recruit GDP-bound Rab5 to the membrane thereby serving as a Rab-receptor (step *b- figure 2*). However, Pra-1 does not exclusively interact with Rab5 but also with other Rab proteins found on other compartments, suggesting that this family of proteins cannot alone determine the specificity of Rab protein localization. In yeast, GEF-proteins were postulated to establish Rab-recruitment cascades: Ypt31/32p (orthologues of mammalian Rab11), for example, was shown to recruit in its active form the GEF-protein (Sec2p) which is required to activate Sec4p (yeast orthologue of Rab8A, Rab8B and Rab10), the Rab GTPase acting immediately downstream of Ypt31/32p (Ortiz et al., 2002). A similar cascade was described for the Rabs Ypt1p and Ypt31/32p (Wang and Ferro-Novick, 2002). Such cascades in which one Rab activates the one immediately downstream may prove an elegant method of linking individual Rab domains which are

functionally coupled in space and time. However, GEF, GAP, GDF and GDI family members, components directly regulating the Rab cycles are in general promiscuous and never specific for one particular Rab. It therefore remains enigmatic what restricts the localization of Rab proteins to distinctive intracellular compartments.

Upon delivery to the endosome, GTP-exchange factors (GEFs), such as the Rabex/Rabaptin-5 complex convert Rab5-GDP to its GTP-bound or 'active' form (see figure 2). Interestingly, in the case of Rab5, the 'active' form of Rab5 can recruit the Rabex/Rabaptin-5 complex generating a positive feed-back loop which amplifies the generation of more GTP-bound Rab5 from a single 'seed' of active Rab5 molecules (Lippe et al., 2001). Besides Rabex, other VPS9-domain containing enzymes such as Rin3 and Alsin (Als2) were found to catalyze the exchange of GDP for GTP on Rab5 (Kajiho et al., 2003; Otomo et al., 2003).

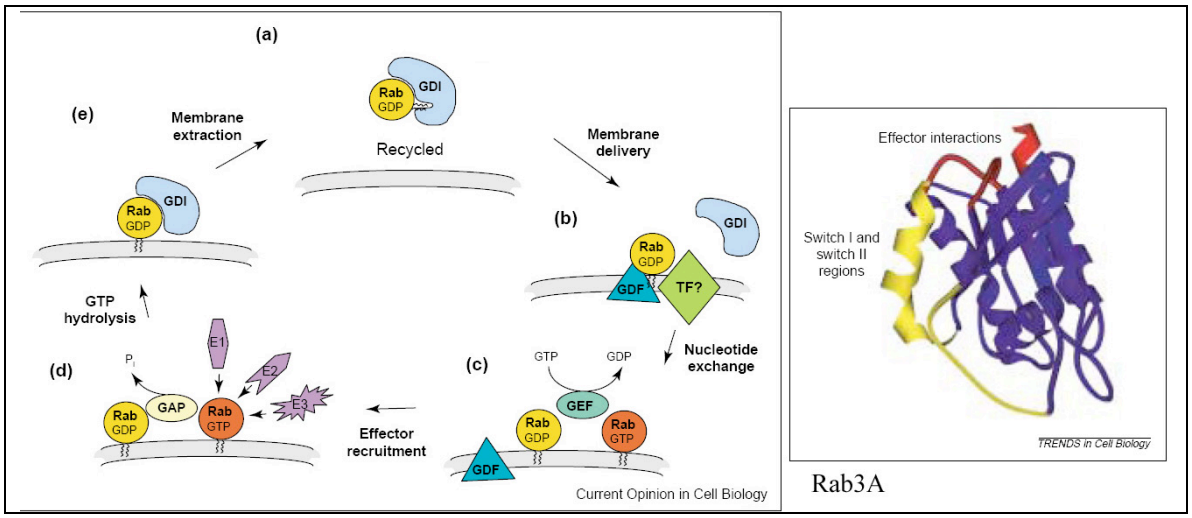


Figure 2:
On the left: ***Schematic representation of the Rab cycle showing membrane recruitment and activation.*** *(a) GDP-bound Rab proteins form a cytosolic complex with RabGDI. (b) Membrane delivery and RabGDI displacement are mediated by a GDF, probably aided by unidentified targeting factors (TF), followed by (c) Rab activation through GEF-catalysed nucleotide exchange. (d) GTP-bound Rab recruits effector molecules to the membrane. (e) GAP-mediated GTP hydrolysis returns the Rab to its inactive state, resulting in re-extraction from the membrane by GDI. (modified from (Seabra and Wasmeier, 2004)).*
On the right: ***Crystal structure of Rab3A with highlighted effector binding surfaces (in yellow and blue).*** *Figure adapted from (Pfeffer, 2001b).*

Analogous to the ras oncogene, the intrinsic GTPase activity of Rab enzymes is relatively slow but can be accelerated through GTPase-activating-factors (GAPs). In the case of early endosomes, RN-tre (Lanzetti et al., 2000) and other molecules such as PRC17 (Pei et al., 2002) function as GAPs stimulating the conversion of Rab5-GTP to Rab5-GDP. In its GDP-bound form Rabs are thought to be prone to extraction from the membrane via the GDI protein. For Rab3, this process is facilitated by the chaperone HSP90 (Sakisaka et al., 2002). A crystal structure of Rab3A exemplifies two intra-molecular surfaces within Rab-molecules which are believed to specifically interact with effector molecules: first, the switch I&II region (yellow) which undergoes major conformational change upon GTP hydrolysis (figure 2)

Second, the X-ray structure of a Rab3A/rabphilin complex revealed that the effector bound near the N-terminus of the rab molecule termed the complementarity determining region (CDR in blue). Structures of GMP-PNP (a non-hydrolysable GTP analogue) bound rab proteins revealed that the CDR region is unaffected by the conformational changes of the switch regions, indicating that effector proteins must simultaneously interact with the conserved switch- (yellow) and the variable CDR region (blue).

Several Rab5-effectors prove to be multimodular, i.e. harbouring a multivalent binding potential allowing these proteins to interact simultaneously with different Rab-GTPases or also Rab GTPases and phospholipids (see below). In recruiting various downstream effector-molecules, Rab proteins such as Rab5 can form multi-functional domains which mediate membrane tethering and fusion events (discussed later), can convey organelle motility and cargo sorting. Alone for Rab5, over 30 effector proteins have been isolated so far ((Christoforidis et al., 1999a; Christoforidis et al., 1999b)) covering a wide range of potential enzymatic functionality which can be implemented on an active Rab5 domain. As a consequence, Rabs together with phosphoinnositide lipids assemble complex networks of effectors on the endosomal surface thereby coordinating specialized functionality in space and time.

Amongst the many effectors of Rab5 are also phosphoinnositid kinases (Christoforidis et al., 1999b) and phosphatases (H.Shin et al. manuscript submitted) which generate and maintain phosphatidylinnositol-(3)-phosphate (PI(3)P) on the early endosome while hydrolysing other phosphatidylinositol species.

V. III. 2. Lipids in endocytic compartments

Phospholipid generation and consumption is regulated by various lipid kinases and phosphatases which are integral components of various intracellular organelles and the plasma membrane. Figure 1 outlines the identities and location of various lipid species throughout intracellular compartments at steady state. Lysobisphosphatidic acid (LBPA), for example, is found to accumulate in the membranes of internal vesicles of late endosomes/multi vesicular bodies, where it has been postulated to regulate membrane curvature and membrane fusogenicity (Kobayashi et al., 2002).

As mentioned earlier, lipids also aid in protein sorting. Entry into the cell via caveolae, for example, involves partitioning of cargo into liquid ordered "rafts", lipid microdomains featuring a particular high content in sphingolipids and cholesterol (Simons and Ikonen, 1997). Membranes enriched in rafts circulate between the plasma membrane, early- and recycling endosomes (Miaczynska et al., 2004b). Proteins which preferentially segregate into these microdomains include GPI-linked proteins and double-acylated proteins (Toomre et al., 2000) but not Rab proteins (unpublished observation) which are generally considered suitable 'non-raft' markers.

A total of seven phosphoinositides have been found in cells which are generated by various kinases (see figure 3). In eukaryotes, PI(4)P and PI(4,5)P2 represent the two most abundant phosphoinositides, although they total only 0.5% of all lipid membranes (Takenawa and Itoh, 2001). These phosphoinositides are generated at the Golgi and the plasma membrane, while PI(3)P is abundant on early endosomes, macropinosomes and phagosomes (compare figure 1). PI(3,4,5)P3 is mainly synthesized at the plasma membrane where this lipid is involved in macropinocytosis and the generation of the

phagocytic cup. The localized production of phosphoinositides on organelles can be sufficient to recruit proteins harboring conserved lipid binding motifs such as PhoX, Enth, Fyve, PH, C2 and ANTH domains. Lipid recognition by these binding motifs can be highly precise: Fyve-finger containing proteins, for example bind specifically to PI(3)P which is enriched in early endosomes. In fact, several Rab5-effector molecules feature, in addition to their Rab5 binding site, a fyve-domain, thereby fine-tuning their selectivity in binding to Rab5/PI(3)P domains on early endosomes (for reviews on phospholipids metabolism and lipid-protein interaction see: (De Matteis and Godi, 2004; Lemmon, 2003)).

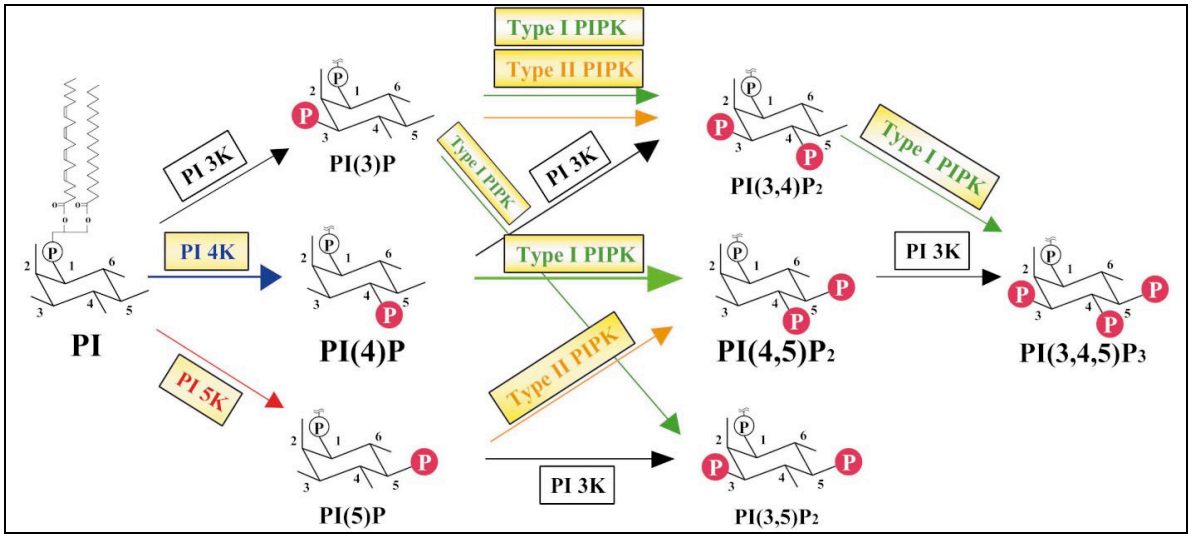


Figure 3:
Phosphoinositides and their biosynthesis.
All 7 phosphoinositides are synthesized from phosphoinositide (PI). Type I PI(4)-5-kinases and type II PI(5)-4-kinases encompass three subtypes each. Nine members are known for the PI(3)kinase family. Figure taken from: (Takenawa and Itoh, 2001)

On early endosomes, active Rab5 recruits the type III PI(3)-kinase vsp34 that generates, in conjuction with its regulatory subunit p150, the majority of the PI(3)P present on early endosomes (Christoforidis et al., 1999b). The remaining pool of PI(3)P is generated by the endosomal PI(4)- and PI(5)-phosphatases INPP4A, INPP5PF and INPP5B (H. Shin, et al.. manuscript submitted). Rab5 also interacts with another PI(3)-kinase, the type I PI(3)K, p110β/p85α. In contrast to vps34, the type I kinase p110β/p85α is localized to the plama membrane where it produces PI(3,4)P2 and

PI(3,4,5)P3 (Sotsios and Ward, 2000). Rab5 may therefore regulate the production of different phospholipids in two distinct compartments. Clearly, these kinases are recruited to their respective target membranes not solely by Rab5 but also by secondary binding partners which must be specifically localized to the endosome or the plasma membrane. In fact, phosphotyrosine containing proteins, such as the PDGF receptor, for example, recruit the catalytic subunit p110beta to the plasma membrane through the regulatory subunit p85alpha which directly interacts with the receptor via an SH2 domain (Okkenhaug and Vanhaesebroeck, 2001).

Despite exhibiting a higher promiscuity in their binding specificity, PhoX(PX)-domains typically also bind PI(3)P on early endosomes. The presence of a PX domain is also hallmark of all 25 members of the *sorting nexin* (SNX) family allowing these proteins to bind PI(3)P, PI(3,4)P2 and, in some cases, also PI(3,4,5)P3. In mammals, besides interacting with phosphoinositide, sorting nexins also frequently affect the sorting and turnover of various receptors such as EGFR (SNX-1, SNX-2, SNX-9, SNX-13), LDLR (SNX-17), and PDGF and transferrin receptor (SNX-15), for example (Worby and Dixon, 2002). Interestingly, so far no sorting nexin has been reported to directly interact with Rab5.

Besides membrane trafficking, phosphoinositides also serve as substrates to lipases generating second messangers such as inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DG) that are important in signal transduction cascades. Furthermore, in response to extracellular stimuli the generation of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) induces the reorganization of actin filaments causing a change in cell shape (for review see (Takenawa and Itoh, 2001)). In conclusion, it should be pointed out that evidence still is still too scarce to conclude if lipids affect the localization of Rab proteins or, conversely, if Rab proteins can modulate their lipid environment by activating phospholipid-kinases directly. As will be explained in the following chapter, phosphoinositides also function in coat assembly by recruiting various adaptor molecules.

V. IV. Coats

The current concept behind carrier vesicle mediated transport in membrane traffic can be summarized in a 7-step process as illustrated in Figure 4 (Bonifacino and Glick, 2004) Initially, cargo is concentrated in the donor compartment (1), coat proteins, and adaptor molecules (see below) are assembled on the cytosolic face of the membrane inducing membrane curvature and the formation of a bud (2). Next, the forming vesicle fissions from the donor compartment (3) to subsequently uncoat (4). The uncoated vesicle is now transported to the target membrane. This process may involve components of the cytoskeleton and molecular motors. Following reversible thethering (5) the vesicle can finally dock (6) and fuse with the target organelle (7). Tethering and fusion processes will be described in section V.IV.

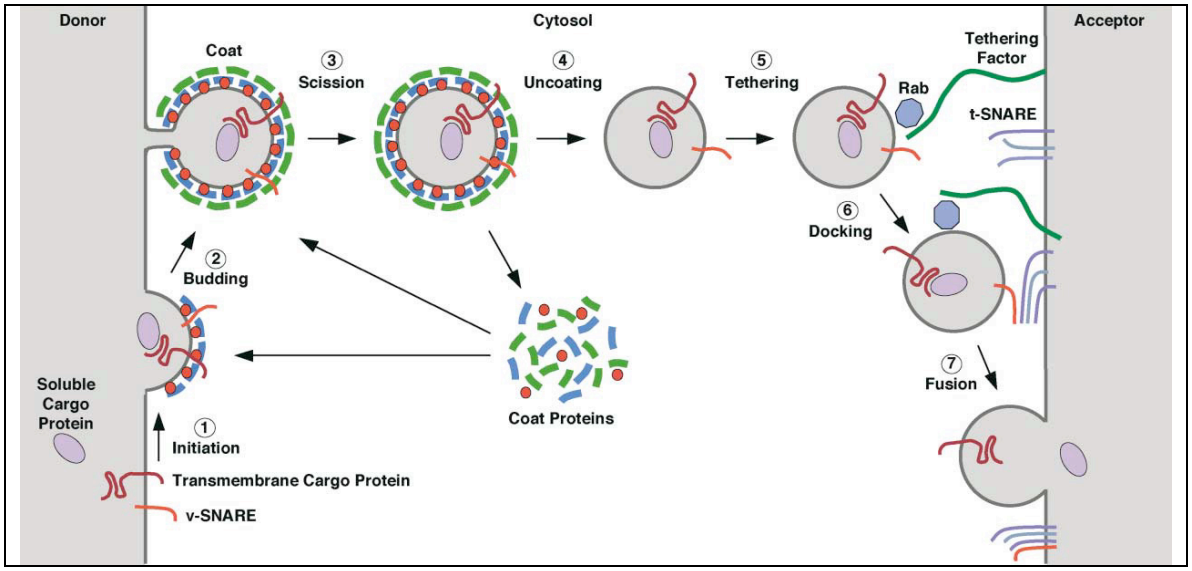


Figure 4
Vesicles can transport soluble cargo and transmembrane proteins between two non-identical compartments.
The 7 steps involved in vesicle mediated transport are shown. Figure taken from: (Bonifacino and Glick, 2004).

V. IV. 1. Clathrin

Clathrin-dependent endocytosis involves the polymerization of clathrin molecules into clathrin lattices. These lattices consist of clathrin triskelions which in turn are trimers

of clathrin heavy chain proteins (~190kDa) and clathrin light chains (~25kDa) (see Figure 5-A). Recent X-ray diffraction studies resolved clathrin lattices at subnanometer resolution (Fotin et al., 2004) providing a detailed model of how 36 triskelions could assemble into a hexagonal barrel. Interestingly, the cytoplasm contains a significant amount of pre-assembled triskelions. Since preformed clathrin triskelions can rapidly self-assemble into lattices *in-vitro,* their polymerization *in vivo* must be tightly regulated. The assembly of the electron-dense coat during endocytosis is nicely observable using transmission electron microscopy. Figure 5-B depicts TEM snapshots of the bud formation process (Alberts B, 2004). The cartoon in Figure 5-B illustrates how clathrin, adaptor molecules such as AP-2 and AP-180 (discussed later) and accessory molecules assemble into a functional vesicle coat. While clathrin is utilized by the cell at various endocytic transport steps, two additional coatomer proteins termed *COP-I* and *COP-II* function in vesicle formation and transport steps between Golgi and the ER. Furthermore, the yeast proteins Vps5p/Vps17p of the retromer complex (Pfeffer, 2001a) also act as a self-organizing coat around vesicles that probably form to retrieve cargo from the prevacuolar/late endosomal system back to the Golgi. It remains a mystery at which point of the pathway and which cargo the retromer sorts and transports in mammalian cells (Pfeffer, 2001a).

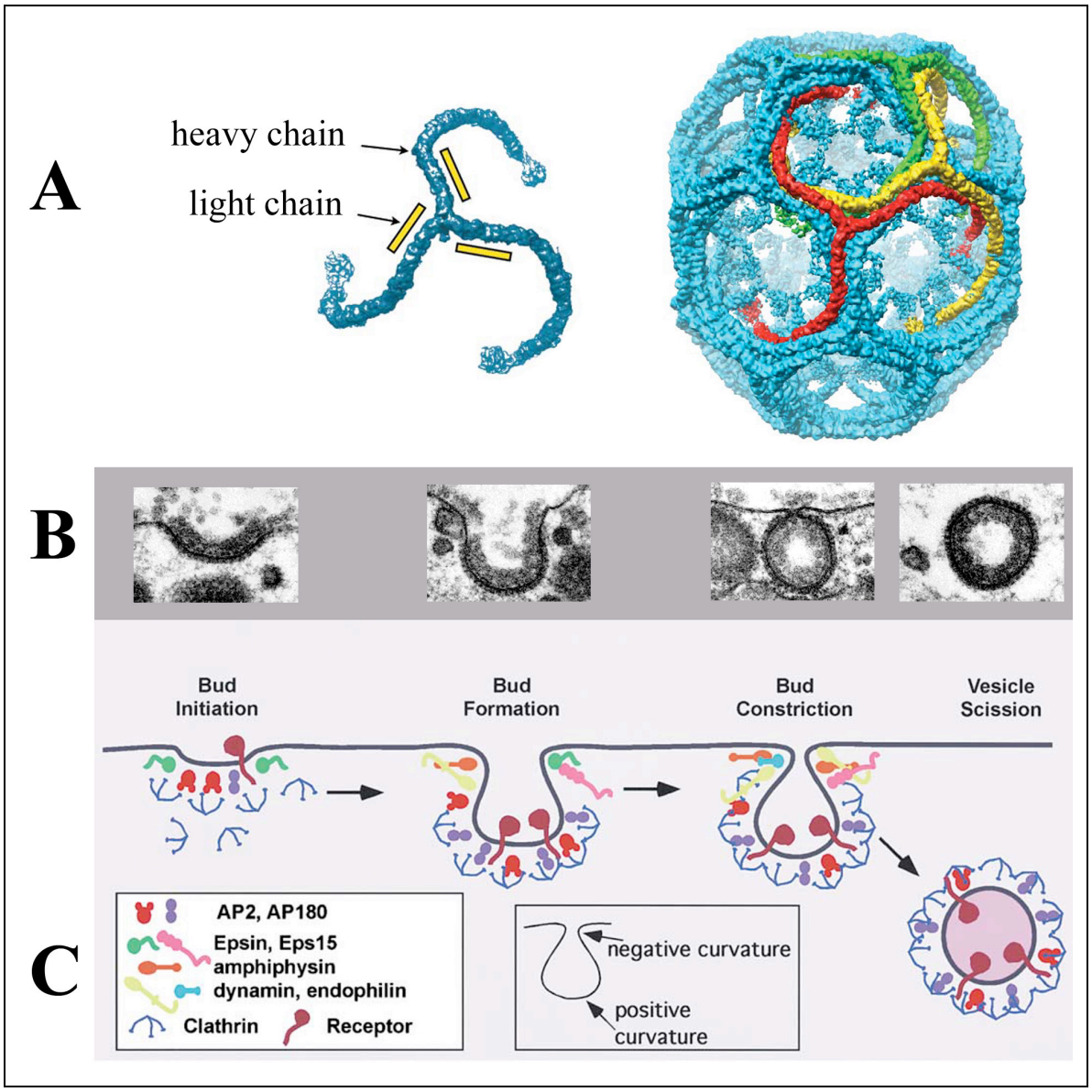


Figure 5
The formation of a clathrin coated vesicle (CCV)
(A) Three light and three heavy chains of clathrin form a triskelion. 36 Triskelions can self-assemble into a hexagonal barrel. (B and C) Snapshots using transmission electron microscopy (TEM) illustrating the transition of clathrin coated buds into clathrin coated vesicles. A cartoon provides an overview of some adaptor and accessory proteins involved during this process (C). Images are taken from: (Alberts B, 2004; Fotin et al., 2004; Hurley and Wendland, 2002)

V. IV. 2. Accessory proteins mediate membrane curvature and fission

To form a nascent vesicle, the lipid bilayer must undergo bending. The forces necessary for the physical distortion of the membrane are thought to originate from a)

clathrin polymerization b) dynamin and accessory proteins and c) proteins harboring *epsin-N-terminal-homology* (ENTH) domains. Since clathrin binds indirectly to phospholipids via adaptor proteins, membrane curvature could be a direct result of the triskelion oligomerization process. However, flat clathrin lattices have been observed on early endosomes (Sachse et al., 2002), indicating that clathrin is probably not sufficient to induce membrane curvature by itself. In addition, upon treatment of nerve terminals with GTPγS, the large GTPase dynamin self-assembles into a helical polymer forming a collar around the neck of membraneous tubules (Takei et al., 1995). This process has originally been proposed to mediate scission of the nascent vesicle form the plasma membrane by translating free energy harnessed through GTP hydrolysis into mechanical cleavage. While dynamin-dependent cleavage has actually been observed *in vitro* (Sweitzer and Hinshaw, 1998), it is still under debate, whether dynamin is sufficient to induce fission *in vivo*. Dynamin interestingly contains its own GAP-domain (termed GED) which promotes GTP hydrolysis following self-assembly. Mutations in this domain can lead to the accumulation of constricted coated pits. Another mutation within the GAP domain rendered dynamin incapable of polymerization while actually stimulating vesicle formation. From these and previous data, the authors argue that dynamin is in deed a 'constrictase' but no true 'pinchase' (Sever et al., 2000). Functioning as a GTPase, dynamin could also serve to recruit downstream accessory molecules, such as endophilin or amphiphysin, for example, which may represent cofactors required to sever the nascent vesicle from the plasma membrane (Huttner and Schmidt, 2002). Figure 6 gives a nice overview over possible models that explain how this mechanochemical enzyme could function in endocytosis. Next to its suggested function in membrane fission, dynamin has recently also been observed to act as a SNARE (see below) filter, proposing a pleiotrophic role for this molecule in affecting not only fission but perhaps also fusion events (Peters et al., 2004).

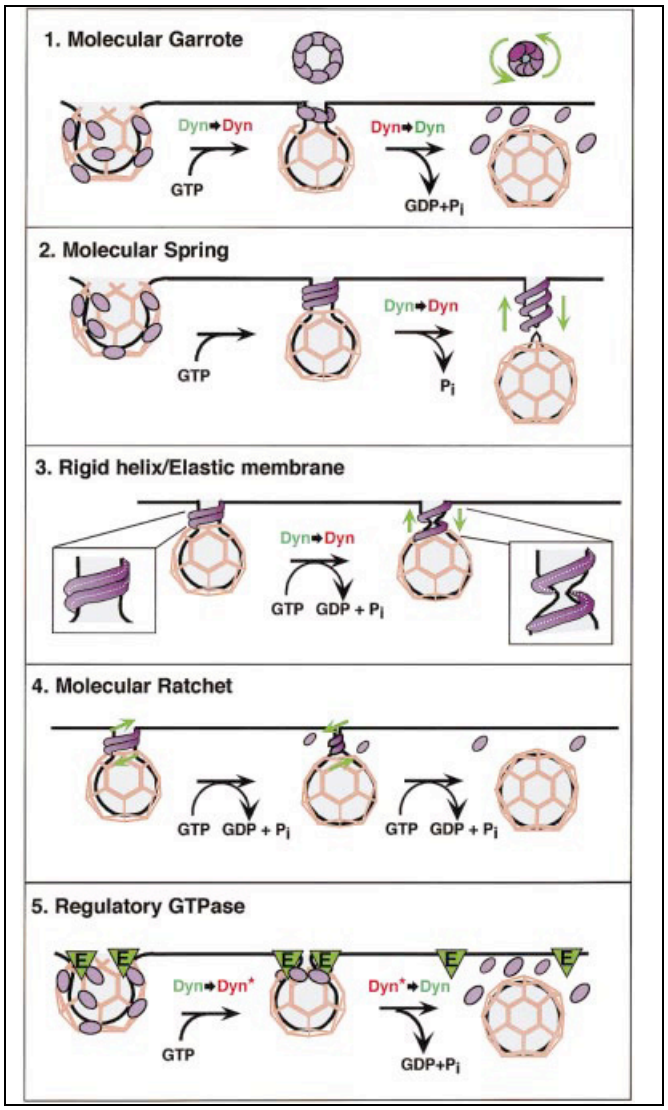


Figure 6
Cartoon illustrating possible mechanisms by which dynamin could, under the consumption of GTP, drive vesicle fission events in the cell. *Image taken from:(Sever et al., 2000)*

One has to realize, that at the state of bud constriction, both positive as well as negative membrane curvatures are present (Figure 5). While dynamin, endophilin as well as amphiphysin were all found capable of tabulating membranes (reviewed in (Hinshaw, 2000)), membrane bending was found to also rely on proteins such as epsin containing the ENTH domain. This domain directly interacts with phosphoinnositide(4,5)P_2 (PIP_2) and can induce membrane tabulation by itself i.e. without the necessity to self-polymerize (reviewed in (Hurley and Wendland, 2002)). Finally, changing the lipid composition

within the membrane by altering the stochiometry of 'cone-shaped' and 'inverted cone-shaped' phospholipids can promote membrane curvature. For example, endophilin and phospholipase A_2 activity has been shown to induce the tabulation of various organelles via lipid conversion (de Figueiredo et al., 1998).

Membrane bending and vesicle fission precedes the uncoating process which will be briefly discussed in the following section.

V. IV. 3. Coat disassembly and recycling

Fusion of a newly formed vesicle with its target organelle is rendered impossible if the vesicle is still coated. Furthermore, the coat components need to be recycled and made available for future budding events. Therefore, cargo-coat interactions are destabilized soon after vesicle budding. In the case of CCVs, three proteins are important for uncoating: the heat-shock protein hsc70, auxilin/GAK and synaptojanin. First, clathrin-bound auxilin recruits hsc70 and subsequently stimulates its ATPase activity. Upon ATP hydrolysis, hsc70 disassembles the clathrin coat and auxilin is released to cytosol. Meanwhile, synaptojanin 1 (for review see: (Stenmark, 2000)) is thought to hydrolize PI(4,5)P_2 into PI(4)P thereby causing the release of the PIP_2-interacting adaptor protein AP-2 (see below) together with clathrin. As discussed earlier, phospholipid conversion also assures the preservation of membrane identity and vectoriality of transport. Hence, PI(4)P will be converted to PI(3)P on endosomes or to PI(4,5)P_2 and PI(3,4,5)P_3 on the plasma membrane (also see Figure 1). It is noteworthy to mention that both PIP_2 and synaptojanin have been also linked to pathways that regulate the polymerization of actin. The role of actin and microtubules will be discussed in more detail in section *V. IX.*

V. IV. 4. Coat components share structural features with nuclear pore proteins.

A recent comparative structural analysis (Devos et al., 2004) of coat components and nuclear pore proteins allows interesting speculations about how modern-age membrane traffic might have evolved from a more primitive tubular

compartmentalization of endomembranes (Figure 7). On the basis of structural similarity between some key nuclear pore components (see figure 7) and coat proteins typically required for intracellular traffic pathways it was suggested that ancient membrane bending molecules (also see V. VI. 2.) represent the evolutionary precursors to both coat and nuclear pore components. Early endosomes are highly motile organelles which traverse cells by recruiting actin- and microtubules-dependent molecular motors (see below). Endosome motility can be strongly compromised by the depolymerization of microtubules or by overexpression of the small GTPase RhoD which tethers early endosomes to actin cables (Gasman et al., 2003). Interestingly, under these conditions, neither transferrin uptake nor recycling is noticeably inhibited ((Apodaca, 2001), Tobias Heckel and Sebastian Hoepfner - unpublished observation), leading to the intriguing question how much of the endocytic system might still be interconnected through a network of tubular endomembranes (figure 7; see also: (Frolov et al., 2003)). As mentioned earlier, endocytic compartments are acidified by proton pumps. It remains unclear if the pH gradient through the endocytic network reflects a gradient in activity/concentration of the proton pumps in these compartments or if truly enclosed membrane compartments contain and enrich protons over the time of endosome maturation.

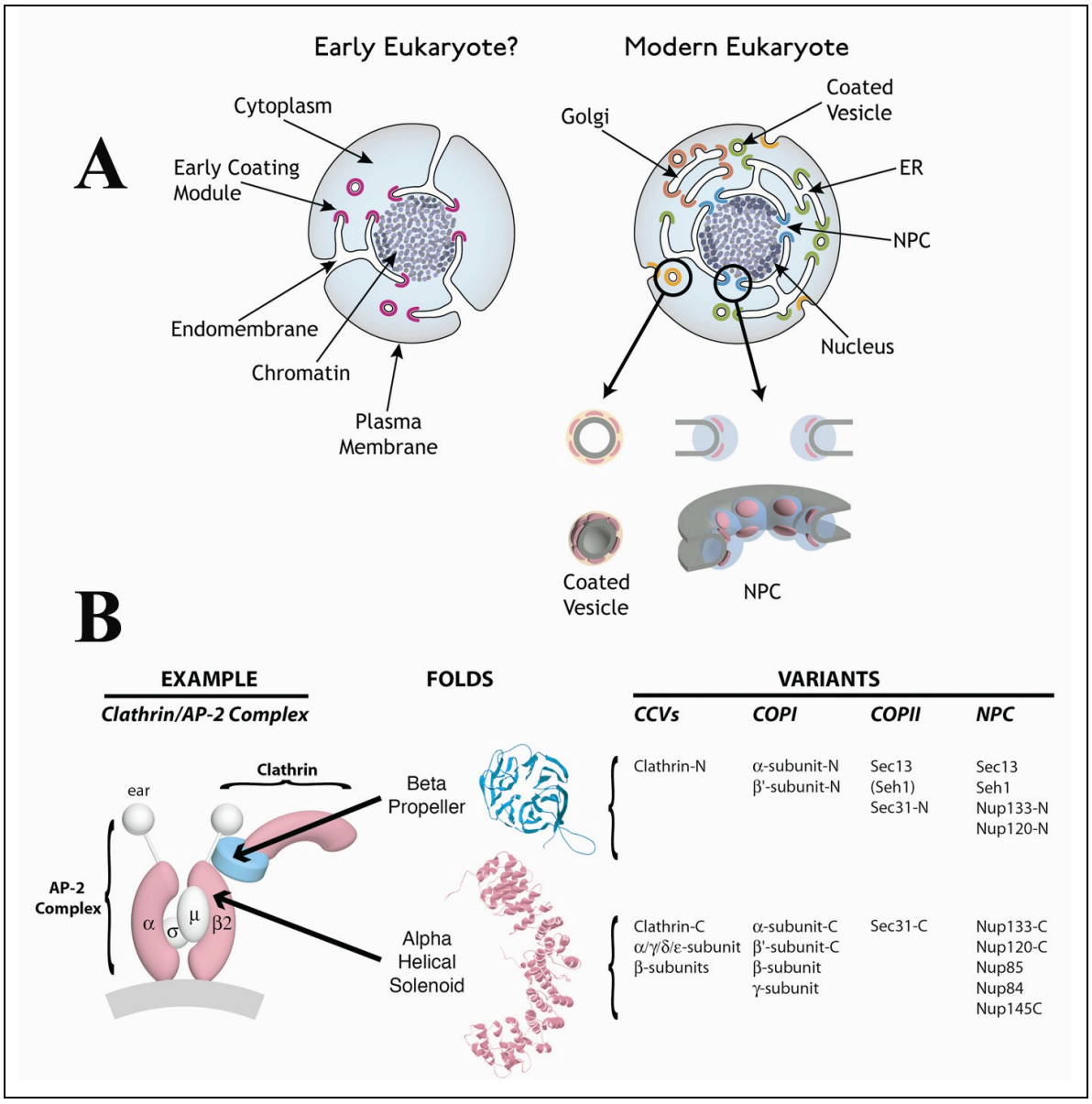


Figure 7
Model illustrating the co-evolution of coated vesicles and nuclear pore complexes.
(A) Archaic eukaryotic cells acquired proteins which induce membrane curvature initially forming a simple network of internal membrane channels. Much later, evolution has segregated this membranous network into distinct organelles such as endosomes, Golgi and ER. (B) Components assembling the nuclear pore complex (NPC) and coated vesicles (CCV, COPI, COPII) share common structural features (folds). The alpha helical solenoid (pink) of the beta-2 subunit of the heterotetrameric AP-2 complex is shown here binding the N-terminal beta-propeller (cyan) of clathrin heavy chain. Figure adapted from: (Devos et al., 2004)

V. V. Principles of Cargo Sorting

Without effective sorting mechanisms, the dynamic exchange of cargo between compartments would soon lead to complete intermixing of lipid and protein composition between organelles and thereby to the destruction of compartment identity. The following section will provide an overview over key sorting mechanisms that are required for proper cargo sorting and maintenance of non-identical compartments within the endocytic network.

V. V. 1. Adaptor molecules and sorting signals

Cargo molecules are sorted with high but not absolute fidelity to their correct destination. Coat and adaptor molecules play an important role in concentrating cargo prior to transport. During coat assembly, adaptor molecules can differentiate between types of cargo by selectively interacting with certain transport motifs (signal for ER translocation, for example) present within each cargo molecule (see figure 8). The interplay between clathrin triskelions, accessory molecules and adaptor proteins during endocytosis is depicted in figure 5. Key adaptor proteins include the heterotetrameric AP1, AP2, AP3, AP4 complexes and the monomeric ***G****olgi localized, ***γ****-ear-containing* ***A****rf-binding proteins* (GGAs), *disabled-2* (Dab-2), *hypercholesterolemia protein* (ARH), ***h****epatocyte growth factor* ***r****egulated tyrosine kinase* ***s****ubstrate* (HRS) (Raiborg et al., 2001), Epsin1, Eps15, HIP1/Hip1R and AP180/CALM (Aridor and Traub, 2002). The adaptor complex AP-2 functions early in cathrin mediated endocytosis. Figure 7 illustrates the typical heterotetrameric structure of AP molecules taking AP-2 as example. Loss-of-function studies with AP-2 involving either AAK1-dependent clustering or silencing of AP-2 components via RNAi hardly affected endocytosis of EGF- or LDL-receptors but strongly reduced the formation of clathrin coated pits and inhibited trasferrin receptor internalization. Interestingly, cells remained perfectly viable despite non-functional AP-2 adaptor complexes (Robinson, 2004). Like AP-2, most other adaptor molecules bind clathrin, cargo and additional accessory proteins via multiple, low-affinity interactions. For example, both Dab2 and ARH adaptor molecules colocalize well with AP-2 and function in clathrin mediated budding processes, yet recognize different

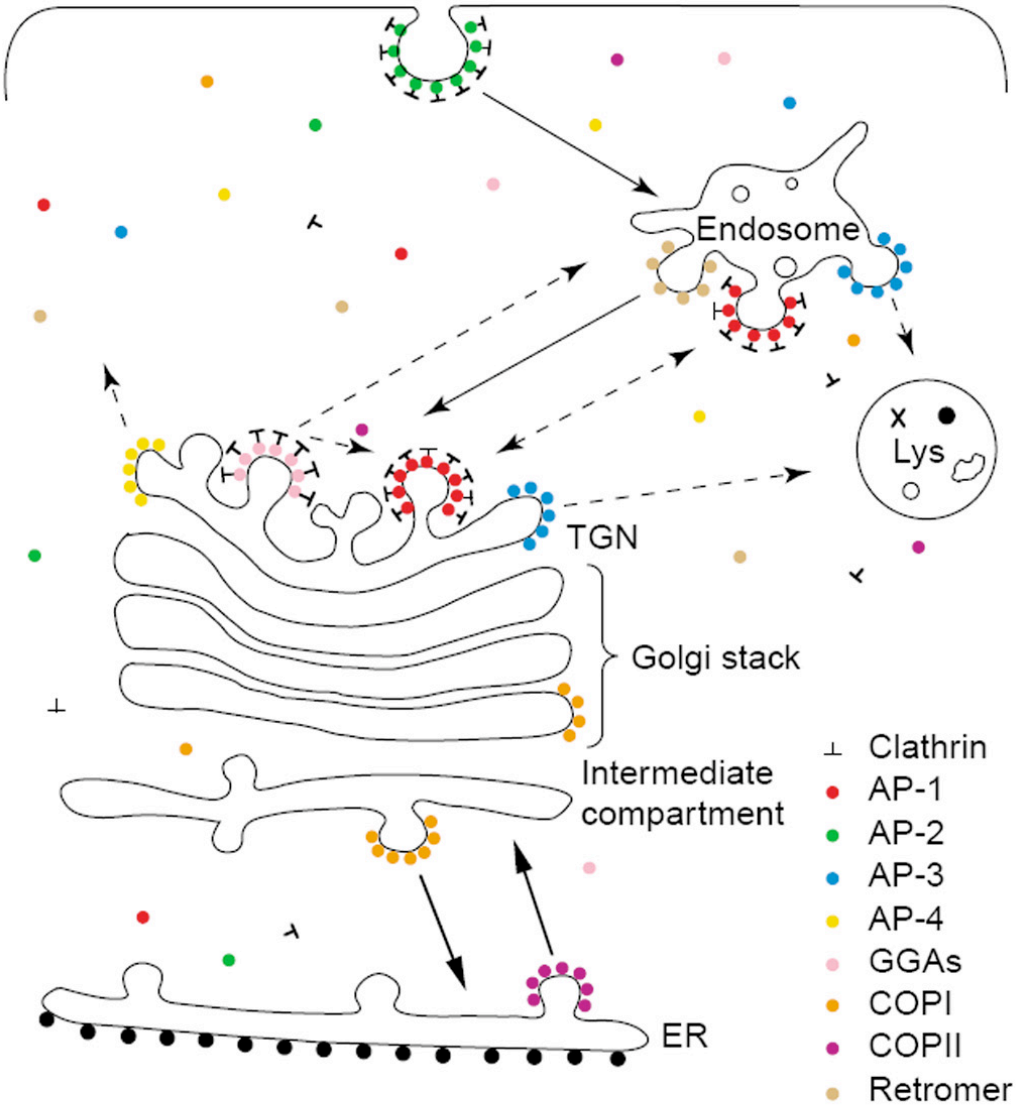
transport motifs within their cargos. Figure 8 gives an overview which sorting signals and phospholipids preferentially interact with which adaptor molecules and what traffic steps are mediated by each adaptor or coat molecule.

Another property of many adaptor molecules is their ability to bind to lipids. As mentioned earlier, secretory granules emerging from the Golgi usually contain PI(4)P (Panaretou and Tooze, 2002) while vesicles originating from the plasma membrane are enriched in $PI(4,5)P_2$. Differential affinities between specific lipids, coat components and cargo therefore assure that each type of cargo is selectively concentrated in vesicles carrying the appropriate coat. In other words, the type of sorting signal present in the cargo together with the kind of phospholipid moiety present within the budding membrane dictates the type of coat which is assembled around the nascent vesicle. Such mechanism also ensures that cargo concentration precedes vesicle budding preventing 'empty' vesicles from being formed (Kirchhausen, 2000). Since adaptor molecules can also discriminate between folding and glycosylated states of proteins they may also function as quality control agents in post ER traffic by preventing ER resident proteins to enter budding vesicles and secondly assuring that cargo has reached proper folding and glycosylation state.

All members of the large family of proteins known as sorting nexins (SNX) harbour a conserved PhoX (PX) domain which can physically interact with phosphatidylinositol lipids. Individual sorting nexins were also found to interact with clathrin, cargo molecules (such as EGF and TGF-beta-receptor) and other adaptor molecules such as AP1 and AP2 (Lundmark and Carlsson, 2002, Hirst et al., 2003). Therefore, sorting nexins (see (Worby and Dixon, 2002) for review) may represent yet another family of adaptor molecules. It should also be briefly mentioned here that ADP-ribosylation factor (ARF) – GTPases (members of the ras superfamily) play a role in cargo and GGA-mediated coat (AP1/clathrin) recruitment at the Golgi (Boman, 2001). Table 1 summarizes major adaptor molecules most of which are reviewed in detail in (Aridor and Traub, 2002) and in (Robinson, 2004).

Table 1

Adaptor/ Accessory protein	***Function***	***Reference***
AP-1	Clathrin dependent transport between early endosomes and trans golgi network; recruited to TGN via PI(4)P	(Wang et al., 2003)
AP-2	Clathrin dependent transport from plasma membrane to endosomes; binds to PI(4,5)P2	(Collins et al., 2002; Gaidarov and Keen, 1999)
AP-3	Transport from endosomes to late endocytic and/or lysosomes; recruited to early endosomes via PI(3)P	(Peden et al., 2004), T.Baust et al. (submitted)
AP-4	Enriched on TGN; function unclear	
GGA	Transport between golgi and endosomes. GGAs interact with ARFs, Clathrin and cargo. BACE and M6PR have been implicated as cargos	(Doray and Kornfeld, 2001) (He et al., 2005)
Disabled-2	PI(4,5)P2 and clathrin dependent adaptor molecule for members of the LDL receptor family enriched in endocytic structures; highly expressed in kidney; binds myosin VI	(Mishra et al., 2002a; Morris et al., 2002)
ARH	Clathrin dependent adaptor for members of the LDL receptor family	(Mishra et al., 2002b)
HRS	Clathrin and PI(3)P dependent sorting of ubiquitylated epidermal-growth-factor receptor (EGFR) into multivesicular bodies	(Gruenberg and Stenmark, 2004)
Epsins	Interact with PI(4,5)P2, AP-2 and clathrin. Eps15 is implicated in recruitment of AP2 to clathrin coated pits at the plasma membrane. Epsin1 and Epsin2 function in internalization of transferrin and EGF receptors.	(Benmerah et al., 1999; Chen et al., 1998)
AP180/CALM	PIP2 dependent sorting of the SNARE synaptobrevin into CCVs	(Ford et al., 2001)
HIP1/Hip1R	PI(3,4)P2 and PI(3,5)P2 dependent; binds clathrin light chain	(Hyun et al., 2004)
Sorting Nexins	Transport between golgi and endosomes.	(Worby and Dixon, 2002)



Interactions of adaptors with sorting signals and phosphoinositides

Receptors	Motif	Binding partner(s)	
Transferrin receptor	YXXΦ	AP-1, AP-2, AP-3, AP-4	(where Φ is a bulky hydrophobic residue)
LE/Lysosome prot.	[D/E]XXXL[L/I]	AP-1, AP-3; AP-2, AP-4?	
M6PR	DXXLL	GGAs	
LDL-receptor	FXNPXY	Dab2, ARH	
PDGFR, EGFR	Ubiquitin?	Epsins 1–3	
	Phosphoinositide		
	PIP2	AP-2, β-arrestin, Dab2, ARH, epsins 1–3, AP180/CALM, Hip1/Hip1R	
	PI4P	AP-1, epsinR	
	PI3P	HRS	

Figure 8 :
Coated vesicle adaptors function in transport steps linking various compartments. *Dotted arrows represent an ambiguity in directionality or identity of the destination compartment. Below, a table summarizes known interactions between phospholipids,*

adaptor molecules and conserved sorting motifs present in many receptors (see text). Image adapted from (Robinson, 2004).

V. V. 2. Sorting as a result of controlled fusion

After cargo concentration, budding and uncoating, transport of carrier vesicles will terminate in a fusion event between the vesicle and its target compartment. The process of membrane fusion is dependent on Rab proteins which can recruit tethering factors such as EEA1 and a class of transmembrane proteins called SNAREs. The interplay between Rabs thethering factors and SNAREs and their function in membrane fusion will be discussed later in the chapter 'membrane fusion'. A discussion of SNARE mediated sorting can be found there as well.

V. V. 3. Geometric Sorting

In the moderately acidified environment within early endosomes, cargo (such as LDL, EGF or iron) detaches from the receptor. Endosomes exhibit typically tubular protrusions emanating from a larger spherical structure (see figure 9). It has been postulated that membrane bound cargo recycles via these protrusions by bulk flow. Since the tubules feature a much higher surface to volume ratio compared with the remaining endosome, membrane bound cargo would recycle preferentially via these tubules while soluble proteins would be retained. Consequently, membrane-bound cargo molecules lacking a specific targeting signal would be sorted to the recycling pathway by default. This sorting process is also referred to as 'geometric sorting' (Dunn et al., 1989)

Figure 9
Freeze-etch electron microscopy image of an early endosome.
Low-density lipoprotein (LDL) conjugated to goldbeads were previously internalized for 5 minutes (image by Dr. J. Heuser).

V. V. 4. Ubiquitin as sorting signal

RING-, HECT- and possibly PHD-domain containing enzymes catalyze the transfer of ubiquitin to the primary amine of specific target proteins. The RING-finger ubiquitin ligase c-Cbl has been shown to play an important role during endoyctosis and downreagulation of receptor thyrosine kinases (reviewed in (Rubin et al., 2005)). First, growth factor binding induces receptor dimerization and auto-phosphorylation. This enables the ubiquitin ligase Cbl to bind a specific phosphor-thyrosine site of the receptor and subsequently mediates ubiquitination of the receptor. During endocytosis, posttranslational ubiquitin modification serves as a quality control mechanism where a single ubiquitin molecule is sufficient to label the receptor as 'ligand-bound'. As will be discussed in the next section, ubiquitinated receptors arriving at early endosomes are sorted to late endosomes. The genome of the Cas NS1 retrovirus encodes for a mutant form of the c-Cbl protein. The expression of viral v-Cbl in the host cell prevents ubiquitination of EGF receptors leading to constant recycling of activated receptors which finally results in uncontrolled proliferation and oncogenesis (Yarden, 2001). Interestingly, clathrin adaptor molecules of the GGA family that localize to the Golgi also intersect with the ubiquitin pathway – implying that GGAs may also function in sorting ubiquitinated cargo (Puertollano and Bonifacino, 2004).

V. V. 5. Sorting into multi vesiclular bodies

Cargo which is destined to be degraded is first sorted into internal vesicles of multivesicular bodies (MVB) before they mature into or fuse with late endosomes. This sorting step is mediated by the adaptor protein HRS and the three multiprotein complexes ESCRT I/II/III (*endosomal sorting complex required for transport*). HRS localizes to PI(3)P on early endosomes via its FYVE motif where it also binds to clathrin and ubiquitinylated cargo. While the role of HRS bound clathrin is obscure, it has been suggested that HRS recruits the ESCRT complexes to early endosomes. The ESCRT complexes act sequentially and sort ubiquitinylated receptors into intralumenal vesicles. For a detailed review about the ESCRT complexes see: (Katzmann et al., 2002). Finally, annexin-2 separates multivesicular bodies from endosomes, and the PI(3)P-5-kinase Fab1/PIKFYVE transforms PI(3)P into PI(3,5)P2 leading MVB biogenesis to completion.

A second round of sorting occurs at the level of late endosomes where mannose-6-phosphate receptors (M6PR) recycle back to the Golgi, MHC class II receptors are sorted to the plasma membrane while bulk cargo will travel to lysosomes. Interestingly, multivesicular bodies can also fuse with the plasma membrane expelling the internal vesicles as 'exosomes'. Many cell types secrete exosomes. While in most cases the physiological role of emitted exosomes is still unclear, cytotoxic T-lymphocytes (CTLs) are known to secrete granzyme and perforin packaged in exosomes to destroy their target cell (Quah and O'Neill, 2000).

V. VI. Membrane fusion

The last steps of carrier vesicle mediated transport involve fusion of a vesicle with the membrane of its target organelle. This process proceeds sequentially through: (1) reversible tethering, (2) irreversible docking and finally (3) fusion of the 'donor' vesicle with the target membrane 'acceptor'. Due to the presence of counteracting electrostatic forces between the membranes the fusion process represents a highly endothermic

reaction. In general, before fusion occurs, tethering to the acceptor membrane is initiated via Rab GTPase - bound tethering factors. In the case of early endosomes, Rab5 recruits the tethering factor EEA1 which is necessary for the homotypic fusion between early endosomes (Christoforidis et al., 1999a) and early endosomes with clathrin coated vesicles (Rubino et al., 2000). EEA1 forms homodimers in solution, features extensive coiled coil structure common to many tethering factors and harbours two Rab5 binding sites at both ends of its rod-like structure, providing target specificity. Since Rab5 is present both on CCVs as well as on early endosomes, EEA1 is believed to bridge both compartments before fusion occurs. Similar to other tethering factors, EEA1 also directly interacts with syntaxin-6 (Simonsen et al., 1999) and syntaxin-13 (McBride et al., 1999) which are components of the fusion machinery (see below). Other tethering complexes include the HOPS multiprotein complex functioning in transport from early to late endosomes, the TRAPP1&2 (TRAnsport Protein Particle) and golgin (Barr and Short, 2003) tethering complexes at the golgi and the exocyst complex involved in tethering exocytic vesicles to the plasma membrane (Hsu et al., 2004).

After a vesicle is tethered to its acceptor membrane, docking and fusion can occur. The core components of the fusion machinery include the N-ethylmaleimide-sensitive-factor (NSF), the soluble, NSF associated protein called alpha-SNAP and the SNAP-receptors (SNAREs). In 1994, Rothman postulated the *SNARE hypothesis* stating that, in analogy of a lock and key system, only the pairing of a single v-SNARE (on the vesicle) with a cognate oligomeric t-SNARE (on the target membrane) complex leads to successful fusion. The t-SNARE complex usually consists of three individual SNARE proteins with the exception of the synaptic t-SNARE complex consisting only of syntaxin and SNAP-25. SNAP25, which is anchored to the membrane via a lipid modification, harbors in fact two SNARE motifs (see figure 10 - 'A'), thereby completing the tripartite nature of the t-SNARE complex. Docking occurs, when the v-SNARE pairs with the t-SNARE complex to form a very stable four helix bundle termed trans-SNARE complex. Upon membrane fusion, the previously formed trans-SNARE complex (also called SNARE-pin) becomes the cis-SNARE complex to which α-SNAP binds (see figure 10 - 'B'). Alpha-SNAP then recruits NSF which unwinds the SNARE complex under ATP

hydrolysis. The dissociation of the SNARE-pin releases the v-SNARE which is recycled for another round of fusion. Cognate SNARE pairs are sufficient to mediate membrane fusion in vitro (Weber et al., 1998) as well as in vivo (Hu et al., 2003) arguing for their functioning as core fusion machinery. Nevertheless, because the *in vitro* fusion reaction utilizing only minimal essential components proceeds more than an order of magnitude slower than *in vivo* fusion processes (Weber et al., 1998), other components have been postulated to aid or even mediate membrane fusion. The yeast Vo-subunit of the vacuolar ATPase has been proposed to form a fusionpore downstream of the SNARE machinery (Bayer et al., 2003). However, little evidence is provided to show that the Vo-subunit is sufficient to catalyze membrane fusion by itself. Since v-SNAREs are present in anterograde as well as in retrograde (v-SNARE recycling) vesicles, clearly additional accessory proteins such as tethering factors and Rab proteins among others are required to coordinate fusion and to prevent futile fusion cycles. For example, GATE-16 and LMA act downstream of SNAREpin unwinding by binding individual v- and t-SNARE components to keep them separate (Elazar et al., 2003). Furthermore, in the course of tethering and fusion, besides v-SNAREs also Rab proteins will be recycled to the donor compartment.

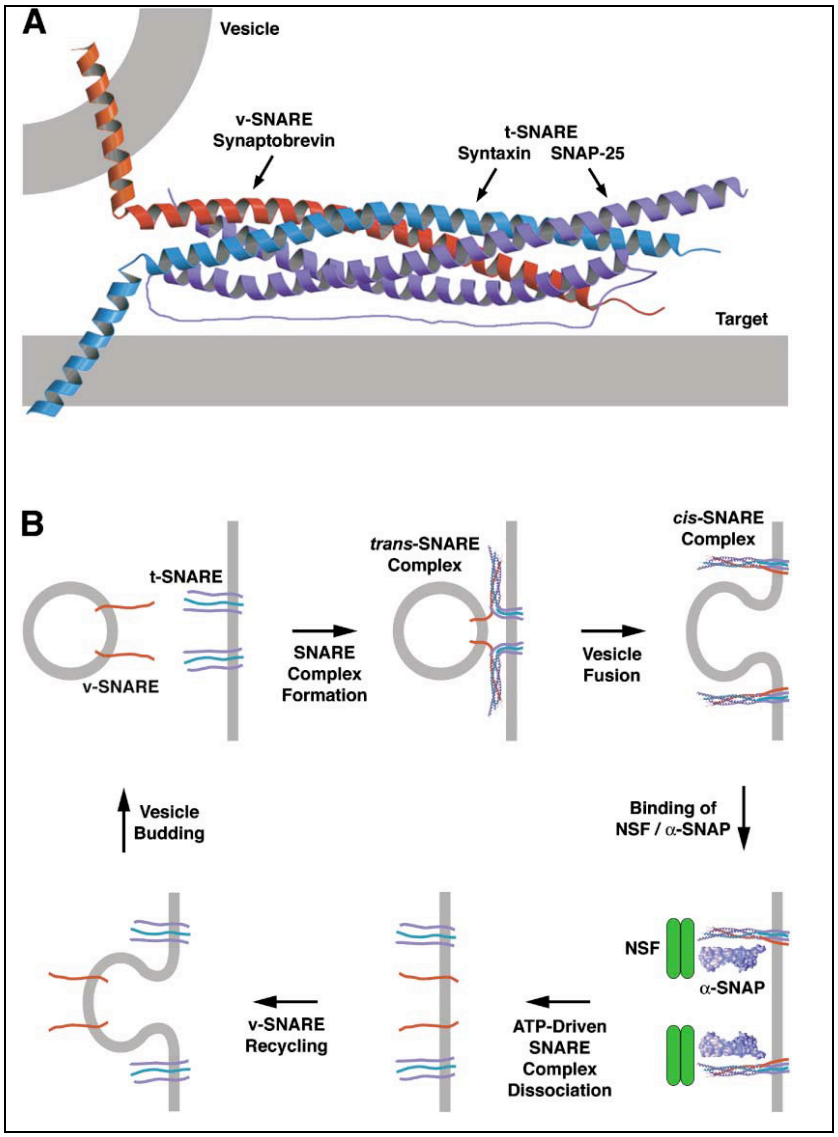


Figure 10:

The structure and function of SNAREs: *(A) pairing of a single v-SNARE with the t-SNARE complex leads to docking and fusion of the cognate lipid membranes. The crystal structure of a trans-SNARE complex is shown (B) The six stages of the SNARE cycle. Please refer to the main text for a detailed description of these processes.*

Figure taken from: (Bonifacino and Glick, 2004).

Different classes of SNARE proteins, much like members of the family of Rab GTPases, are enriched at steady state in specific intracellular compartments (as reviewed in (Hong, 2005)). A vesicle containing a specific v-SNARE will fuse with the

compartment which exhibits the highest concentration of its cognate t-SNARE. Since compartments are constantly exchanging carrier vesicles, efficient SNARE recycling is required to prevent diffusion of SNAREs after multiple rounds of fusion. While adaptor and accessory molecules of coats can sort cargo they also act to filter SNARE-molecules. To maintain compartment identity and prevent SNARE protein mixing, SNAREs are sorted into budding vesicles containing a matching coat type. A mathematical modeling approach has recently been applied to show that such mechanisms could indeed be sufficient to establish compartment identity and regulate compartment size (Heinrich and Rapoport, 2005).

V. VII. Endosomes as platform for Signaling

Another function of early endosomes is to relay and respond to signals coming from the plasma membrane. Since work from this thesis also addresses growth factor receptor turnover, a short introduction will be presented here in how signaling is integrated at the level of the early endosome. Many ligand-bound receptors elicit signaling cascades which are attenuated by the down-regulation (i.e. degradation) of ligand-receptor compexes. A prominent example for this is the EGF receptor. If receptor down-regulation is compromised by naturally occurring mutations, growth factor signaling and tumorigenesis is enhanced. In fact, several signaling pathways regulated by endocytic events directly affect cell proliferation (TGF-beta, APPL) and cell fate determination (NOTCH, SARA) (Miaczynska et al., 2004b; Schweisguth, 2004). Recent FRET studies show that EGF receptors bind to downstream signaling effectors (such as Grb2 and src) on the level of early endosomes (Sorkin et al., 2000) suggesting that signaling continues en route from the plasma membrane to early endosomes. In light of this data, one could just as well postulate that some receptors which recycle through early endosomes might become dephosphorylated by a phosphatase residing on such ‘signaling endosomes’, providing an effective ‘off’-switch for signaling. Finally, silencing of many kinases can lead to striking phenotypes in the context of endocytosis, such as changes in endosome morphology and enhancement or attenuation of clathrin and caveolin/raft-mediated endocytosis (Pelkmans et al., 2005).

V. VIII. Endocytosis in polarized cells

Principles which govern endocytosis in polarized neurons is not discussed here but has been reviewed elsewhere (Sudhof, 2004). In polarized epithelial cells, tight junctions prevent diffusion of lipids and proteins between the apical and basolateral plasma membrane domains. In such cells, endocytosis occurs from both biochemically distinct plasma membranes while material endocytosed apically ends up in apical early endosomes (AEE) and can be recycled via apical recycling endosomes (ARE) while material endocytosed at the basolateral membrane travels to basolateral early endosomes (BEE). Endocytosis form the apical side is regulated by the function of ARF6 and its exchange factor ARNO (Mostov et al., 2000). The small GTPase Rab11 together with one of its effectors RIP11 localize to apical recycling endosomes and are necessary for apical recycling (Prekeris et al., 2000). No true counterpart to the ARE is known to exist at the basolateral plasma membrane. Nevertheless, cargo internalized into BEE (such as the transferrin receptor, for example) can be delivered to the common endosome (CE) and recycle from here back to the basolatral surface. Therefore, the common endosomes is also sometimes referred to as the *common recycling endosome*. This compartment is accessible to cargo independently from which side it has been internalized and represents a pathway intermediate which allows transcytosis to occur. While both AEEs and BEEs harbor the small GTPase Rab5, material endocytosed apically is more likely to be recycled or transcytosed while cargo that enters the basolateral endosomes is most frequently degraded (for overview of traffic pathways in polarized cells see: (Mostov et al., 2003; Mostov et al., 2000)). An exception to this rule is seen in the case of the immunoglobulin IgA. Secreted by lymphocytes in the circulatory system, IgA binds to its receptor (pIgR) on the basolateral plasma membrane of mucosal epithelial cells. Next, clathrin dependent, receptor-mediated endocytosis delivers the pIgR-IgA complex first to BEE then to the CE. From here it is delivered to the apical plasma membrane while it is not clear if this transport step involves the ARE or not. Finally, the transmembrane receptor is proteolytically cleaved to release a complex consisting of IgA bound to part of the exodomain of pIgR into the mucus (reviewed in (Mostov et al., 2003)).

Constant sorting at the level of the AEE, the BEE and the CE is prerequisite to prevent content mixing of apical and basolateral membrane constituents and assures the maintenance of polarity. The cytoskeleton plays an important role to transport cargo with high efficiency between the various endocytic organelles in polarized cells. For example, depolymerization of actin inhibits specifically apical but not basolateral endocytosis. The dynamic interplay between endosomes and cytoskeleton will be discussed next.

V. IX. Cytoskeleton

Motor proteins serve important tasks, such as cargo sorting through membrane tubulation and fission, endosome inheritance and positioning (Goldstein, 2001; Goodson et al., 1997; Hirokawa, 1998). Actin- and microtubule-dependent motors generate the forces required to drive vesicle formation and delivery to their target compartment (Howard et al., 1989). For example, the unconventional Myosin VI motor propels clathrin-coated vesicles through the cortical actin mesh underneath the plasma membrane towards early endosomes (Aschenbrenner et al., 2004). The characteristic intracellular distribution of endosomes results from both short and long-range movements exhibiting a wide range of velocities (Gasman et al., 2003; Nielsen et al., 1999; Valetti et al., 1999). Whereas early endosomes are typically dispersed throughout the cytoplasm, recycling endosomes, late endosomes and lysosomes are situated in close proximity to the nucleus. The minus-end motor Dynein-Dynactin complex is responsible for the perinuclear localization of endosomes and lysosomes (Allan, 2000; Burkhardt et al., 1997) but also for early-to-late endosome transport (Aniento et al., 1993). Plus-end, Kinesin-dependent motility facilitates exocytosis of cargo from recycling endosomes to the cell surface (Lin et al., 2002). Microtubule-dependent motility supports endocytic trafficking between apical early and recycling endosomes as well as transcytosis in polarized epithelial cells (Apodaca, 2001). The kinesin KIFC2 has been proposed to promote minus-ended motility for Rab4 positive, early endosomes (Bananis et al., 2003). However, this kinesin seems to be restricted to neurons, fails to show any phenotype in knock-out mice (Yang et al., 2001) and localizes in its chimeric (KIFC2-GFP) form to mitochondria (J.Rink, unpublished observation). A requirement for microtubule-dependent transport has been

demonstrated neither for endocytosis nor for recycling from early endosomes to the plasma membrane, which rather depends on actin (Apodaca, 2001). The Rab5 effectors RhoD (Gasman et al., 2003), HIP1 (Engqvist-Goldstein et al., 2004), HAP40 (A.Pal, submitted) and members of the HOOK family (Schroer, 2000) all can induce the binding to of early endosomes to actin and might represent regulatory elements which coordinate the switch between actin and microtubule-dependent motility. However, with respect to transport along microtubules, it remains still unclear which molecular motors besides Dynein control the intracellular distribution and motility of early endosomes.

It has become clear that organelles moving bi-directionally along microtubules can switch between plus- and minus end directed motility within milliseconds. How is this switch organized on a molecular level? Is there a continuous tug-of-war between opposing motors or exists a higher order regulatory element which coordinates the decision making process by selectively activating or recruiting only one type of motor at a given time? In the absence of actin, peroxisomes travel along microtubules with a constant step size of ~8nm using the conventional kinesin-1 and the dynein heavy chain (Kural et al., 2005), indicating that these motors are not active simultaneously which would reduce the regular step size. On melanosomes, the bi-directional movement is coordinated by Dynactin which binds both Dynein and the plus end-motor kinesin II (KIF3) (Deacon et al., 2003; Valetti et al., 1999). It remains unknown if motors of opposing polarity bind simultaneously to each organelle and whether diffusion rates would be sufficiently rapid to support directionality switching through differential recruitment of motors from cytosol. Therefore, it remains to be shown by which mechanism these motor activities are regulated.

Increasing evidence points at a role of Rab-GTPases in coordinating organelle motility (Echard et al., 1998; Jordens et al., 2001; Zerial and McBride, 2001). Table 2 lists several GTPases which can directly or indirectly regulate actin and microtubules-dependent motors. As mentioned earlier, on early endosomes, the small GTPase Rab5 coordinates the activity of several effector proteins that cooperatively regulate endocytic trafficking (Zerial and McBride, 2001). A key effector is the phosphatidylinositol-3-OH kinase (PI3-K) hVPS34/p150 that, by generating PI(3)P in proximity to Rab5, allows the

recruitment of Rab5 effectors bearing PI(3)P-binding motifs. The concerted activity of Rab5 and hVPS34 is also required for bidirectional, microtubule-based, endosome motility *in vitro* (Nielsen et al., 1999), raising the questions of which molecular motor(s) may function downstream. It also remains to be shown how the many functional units such as tethering, sorting , fusion and motility can be integrated and kinetically controlled by the GTPase cycle and the spatial segregation within restricted sub-compartments of the early endosome (Zerial and McBride, 2001).

Table 2: links between Rab GTPases and motors

Rab	**Motor**	**Accessory components**	**Organelle**	**Reference**
Rab4?	KIFC2	?	Early Endosome	(Bananis et al., 2003; Yang et al., 2001)
Rab5	Unknown minus end kinesin	---	Early Endosome	(Nielsen et al., 1999)
Rab6	Rabkinesin-6	---	Golgi-derived vesicles?	(Echard et al., 1998; Hill et al., 2000)
Rab GTPases which regulate actin-dependent motility:				
Sec4p	Myo2	?	Secretory vesicles	(Pruyne et al., 1998)ctin
Rab7	Dynein	RILP	Late endosomes	(Jordens et al., 2001)
Rab8?	Myosin Vc	?	Endosomes	(Rodriguez and Cheney, 2002)ells.
Ypt11p	Myo2	?	Mitochondria	(Itoh et al., 2002)

Rab11	Myosin Vb	FIP2	Recycling endosomes	(Hales et al., 2002; Lapierre et al., 2001)
Rab27a	Myosin Va	Melanophillin	Melanocyte melanosomes	(Fukuda et al., 2002; Strom et al., 2002; Wu et al., 2002)
Rab27a	Myosin VIIa	MyRIP	Retinal melanosomes	(El-Amraoui et al., 2002; Gibbs et al., 2003)

V. X. Conceptual outlook

Our view of endocytosis has broadened in the sense that we have improved our understanding of important concepts operating behind membrane trafficking and cargo sorting, domain orchestration through Rabs and phospholipids and membrane based signal transduction. Genetic (D'Hondt et al., 2000) and biochemical screens (Zerial and McBride, 2001) have identified key enzymes which function to maintain organelle identity and vectoriality of transport along the endocytic and secretory pathway. However, despite our insight, we are still lacking a holistic overview over the machinery required for key endosomal functions such as fusion, effector recruitment, cargo sorting or organelle motility. For example, with respect to organelle motility, in vitro systems could successfully recapitulate movement of isolated early endosomes along microtubules. However, the identity of the molecular motors involved remained largely obscure (Nielsen et al., 1999). Clearly, if we hope to understand how Rab domains can implement such plethora of different functionality in space and time, if we want to further our understanding what pathways converge at the level of early endosomes in polarized cells and what impact endosomal signaling has on development and disease, we have to strive to gain a more holistic overview over the enzymatic gears which are necessary to drive the complex processes involved.

The work presented in this thesis is bipartite. In the first part, a bioinformatics screen was devised to search for molecular motors governing early endosome motility. This served

to address important outstanding questions related to how early endosome movement is coupled to cargo sorting and transport. The successful identification of a novel endocytic motor allowed me to address several important questions with respect to endosome motiliy:

Part I: (in silico screen and subsequent in vitro and in vivo studies)

- What molecular motors drive microtubule – dependent, early endosome motility?
- How might bi-directional motility be implemented on early endosomes?
- What physiological relevance has endosome motility and how can molecular motors influence membrane trafficking through early endosomes?

From the work on molecular motors, it became clear that the intracellular distribution of early endosomes is tightly coupled with their function. Yet, a detailed understanding of how endosome motility and positioning is integrated with the secretory, endocytic and recycling machinery in polarized epithelial cells remains elusive. Understanding how polarized cells can generate and maintain distinct plasma membrane domains requires the understanding of the molecular principles underlying the biosynthetic and endocytic trafficking routes, and the structural and functional features of the organelles involved. In part II, *C.elegans* has been utilized as a model system to address the following unresolved questions:

Part II: (Screen in C.elegans enterocytes)

- Why do apical endosomes maintain their polarized localization and which components regulate transport between such endosomes and the cell surface?
- How does a polarized cell regulate the size, shape, subcellular distribution as well as the number of transport vesicles at steady state? A small increase in number of recycling vesicles that bud from a given compartment would result in complete conversion of this organelle into transport carriers over time. On the other hand, a failure resulting in decreased recycling kinetics can result in massive

accumulation of membrane material as is observed in the C.elegans RME-1 mutant (Grant and Hirsh, 1999).

- How do the endocytic recycling pathways contribute to the generation and maintenance of cell polarity?

VI. RESULTS

VI. III. Results part I - Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B

The superfamily of kinesin proteins (KIFs) drive a great variety of microtubule-dependent motility events (Goldstein, 2001; Goodson et al., 1997; Hirokawa, 1998). Each kinesin contains a conserved "motor domain" as well as a variable tail mediating cargo specificity. The Unc104/KIF1A kinesin, which transports synaptic vesicle precursors, utilizes its pleckstrin homology (PH) "tail-domain" to bind directly to PI(4,5)P_2 lipids (Klopfenstein et al., 2002). The finding that Rab5-dependent early endosome motility depends on PI(3)P raises the possibility that this lipid may recruit an adaptor of a motor or, in the simplest case, a motor itself (Nielsen et al., 1999). To date, two distinct conserved structural motifs, the FYVE finger and the PhoX homology (PX) domain, are known to bind PI(3)P (Lemmon, 2003). Here, we searched for motor proteins displaying such motifs.

VI. III. 1. Identification and cloning of the novel kinesin KIF16B

A genome sequence database search was devised to identify candidate kinesins harbouring either a FYVE or PX motif. This search revealed a gene encoding a putative motor protein with a C-terminal PX domain in *D. melanogaster* (DmKLP98A), as well as a previously unknown orthologue in Fugu *T. rubripes* (JGI32205). The paucity of introns in the Fugu genome together with the high similarity of the Fugu kinesin to the human orthologue enabled us to predict the equivalent open reading frame in *H. sapiens*

(KIF16B, a.n. BX647572). To our knowledge, next to KIF1A, KIF16B represents the only other example of an evolutionary conserved kinesin-like protein, bearing a putative lipid-binding domain across all genomes sequenced so far. A cDNA encoding full-length hsKIF16B was cloned and found, in comparison to the BX647572 cDNA, to contain a single nucleotide polymorphism (SNP) at position 820 (G820A). KIF16B is a protein of 1318 amino acid residues with a deduced molecular weight of 152 kDa (Figure 17A). It is predicted to be a plus-end motor protein on the basis of 1) the N-terminal localization of the structurally conserved catalytic domain (amino acids 1-355) and 2) the presence of 4 conserved amino acids C-terminally to the α-6 helix (Figure 17B) (Case et al., 1997).

According to the recent nomenclature (Lawrence et al., 2004), KIF16B is a member of the Kinesin-3 family, encompassing a total of 8 members in the human genome (Figure 17C). Its closest paralogue in *H. sapiens* is KIF1A, sharing 59% sequence identity in the motor domain. KIF16B displays two diagnostic properties of the Kinesin-3 family, a conserved insertion in loop 3 and a fork head homology (FHA) domain in its stalk (Vale, 2003). Kinesins of this family are thought to exist either as monomers like hsKIF1A (a.n.CAA62346) or dimers KHC (a.n.X65873), depending on the presence of coiled-coil motifs (Vale, 2003). Using the Coils algorithm (Lupas et al., 1991), we compared the predicted content in coiled-coil structure of KIF16B with all human Kinesin3 family members, and deduced that KIF16B has a much higher coiled-coil propensity in its stalk, analogous to that of dimeric *D. discoideum* KIF1A and KHC (Pollock et al., 1999)(Figure 17D).

Whereas many KIFs are primarily expressed in brain (Miki et al., 2003), KIF16B is also present in various other organs, e.g. kidney, liver, intestine, placenta, leukocytes, heart and skeletal muscle by Northern and Western blot analysis (see figure 24), in agreement with the broad expression pattern for this gene (C20orf23) reported in the genome-wide expression database *GeneNote* (Shmueli et al., 2003).

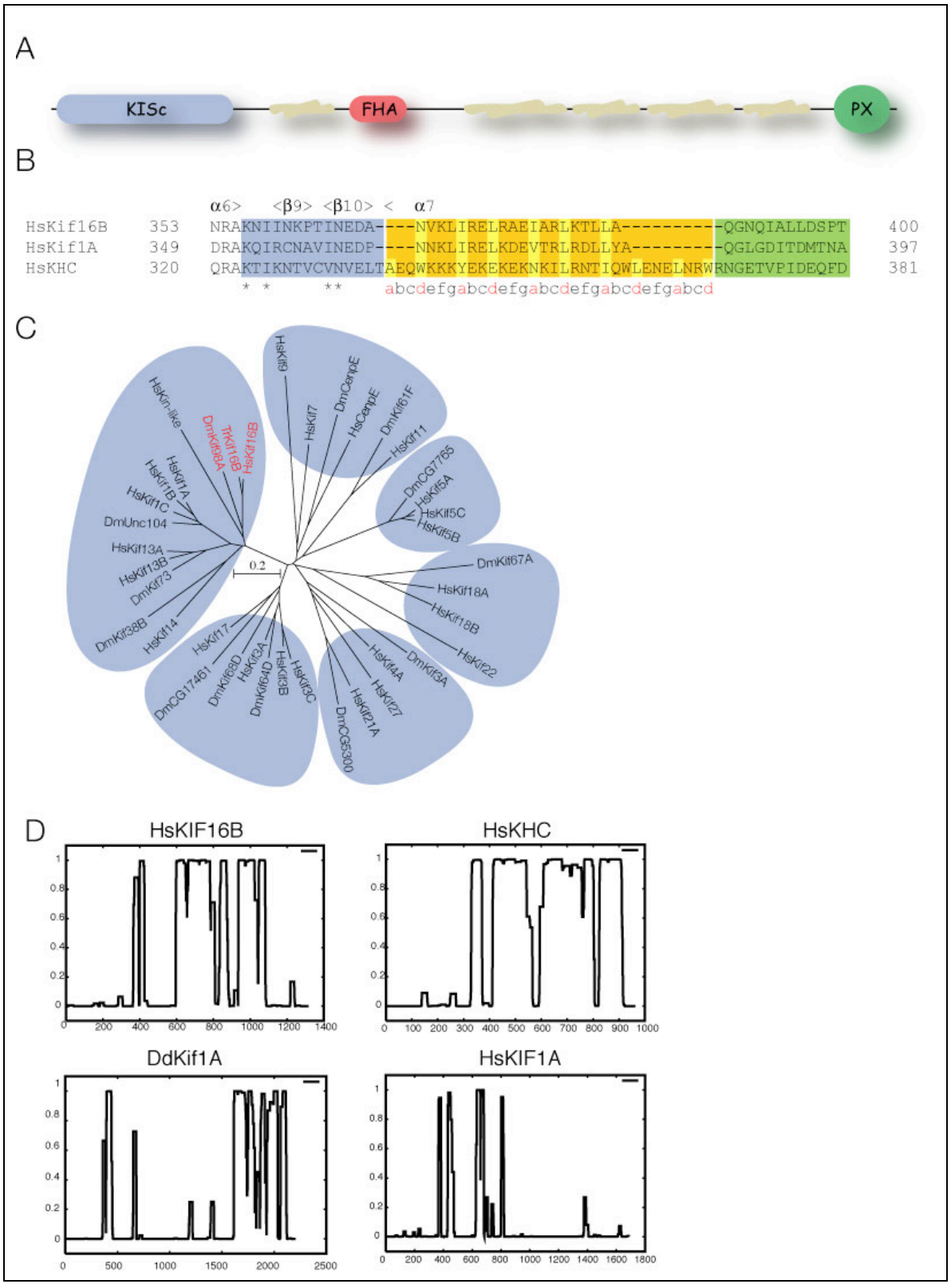
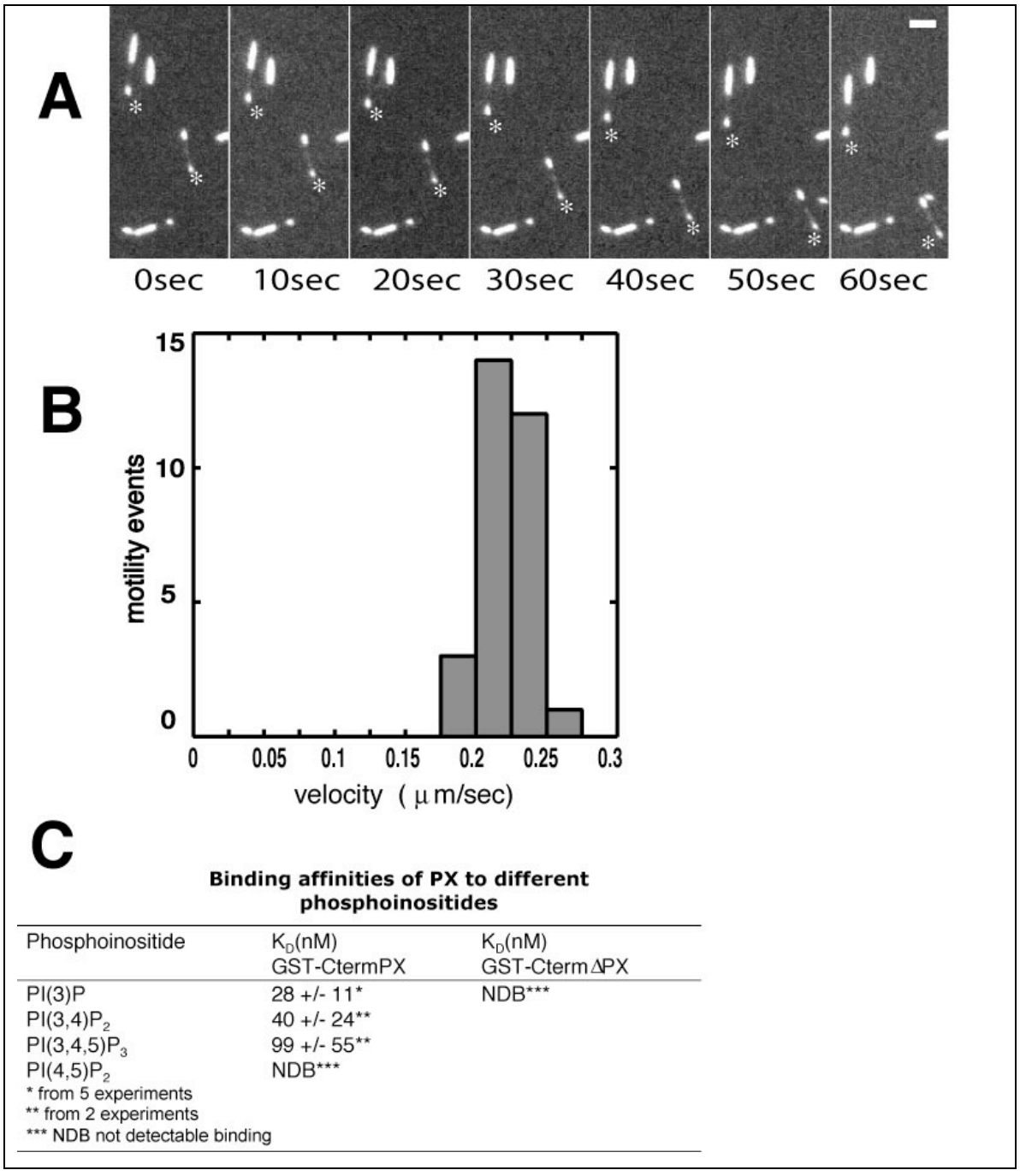


Figure 17. KIF16B, a close paralog to KIF1A, exhibits structural properties of a dimeric, plus-end directed kinesin *(A) Conserved domains within HsKIF16B include the*

N-terminal kinesin motor domain (KISc), the forkhead domain (FHA) as well as the the PX-domain at the C-terminus. (B) The kinesin KIF16B is most closely related to HsKIF1A. (C) The putative neck-linker region (blue), the neck (yellow) as well as part of the hinge region (green) of HsKIF16B was aligned against HsKIF1A as well as HsKHC. The heptad-repeats are very similar to KIF1A. Asterisks (*) mark residues that are well conserved in plus-ended directed motors and predicted β-sheets as well as α-helices are indicated above the alignment. (D) Coils prediction of coiled-coil structures within HsKIF1A, KIF16B as well as KHC. Scores above 0.5 represent significant probability that coiled-coil structures are present.

VI. III. 2. KIF16B is a plus-end directed motor

Recombinant KIF16B expressed and purified from a baculovirus system was tested for the directionality of movement using an *in vitro* assay based on polarity-marked microtubules (Hyman, 1991). In this assay, taxol-stabilized microtubules with bright fluorescent plus ends move on a glass slide coated with the motor protein. In line with our bioinformatics prediction, the majority of microtubules (24 out of 28) were found to glide towards their dimly-labelled minus ends (Figure 18A). Purified KIF16B mediated microtubule gliding with velocities between 0.175 and 0.275 μm/sec (Figure 18B). These values are in the same range of long-distance movements of early endosomes *in vivo*, typically displaying velocities around ~0.3μm/sec (Gasman et al., 2003)(and figure 24). The velocity depends both on the intrinsic properties of the catalytic domain and the stability of the coiled-coil in the neck (Vale, 2003). Although the neck of KIF16B is similar to that of KIF1A (Figure 17C), it differs considerably in the coiled-coil propensity of its stalk (Fig. 17) and in its velocity (~1.20 μm/sec *in vitro* (Okada et al., 1995) and 1.02 μm/sec *in vivo* (Zhou et al., 2001) for KIF1A).



Binding affinities of PX to different phosphoinositides

Phosphoinositide	K_D(nM) GST-CtermPX	K_D(nM) GST-Cterm ΔPX
PI(3)P	28 +/- 11*	NDB***
$PI(3,4)P_2$	40 +/- 24**	
$PI(3,4,5)P_3$	99 +/- 55**	
$PI(4,5)P_2$	NDB***	

* from 5 experiments
** from 2 experiments
*** NDB not detectable binding

Figure 18. KIF16B is a processive, plus-end directed motor and binds PI(3)P through its PX motif *(A) Video microscopy was used to capture movements of labelled microtubules over a lawn of kinesin molecules on a glass slide. Each microtubule carries a short stretch of fluorescently labelled tubulin on its minus-end. The still images shown reveal movement of microtubules in direction of their minus ends (*), driven by the plus end motility of the kinesins. (B) Velocity distribution of the microtubules in (A). Each recorded motility event represents microtubules continuously moving for at least 3 consecutive frames without switching direction (C) GST–KIF16B-PX protein was*

injected into plasmon resonance sensor chips loaded with liposomes containing 2 mol% of each PI. An average K_D was obtained for each PI. No binding (signal below baseline level i.e. less than 2 RU) was observable for GST protein alone and GST-KIF16B-PX and PI(3,4)P2.

VI. III. 3. KIF16B is localized to PI(3)P-containing early endosomes *in vivo*

A key feature of KIF16B is the PX domain at the C-terminus that could target the motor to early endosomes via binding to PI(3)P. We measured the binding kinetics of the PX-domain of KIF16B fused to GST (GST-CtermPX) by plasmon-resonance using a sensor chip coated with different PIs (Figure 18C). Whereas the PX domain of KIF16B did not bind to PI(4,5)P2, unlike KIF1A and Unc104 motors (Klopfenstein et al., 2002), it bound to PI(3)P, PI(3,4)P2 and to PI(3,4,5)P3 with affinities (K_D=28nM, 40nM and 99nM, respectively) comparable to those reported for the FYVE domain of Hrs binding to PI(3)P (K_D of 38 ± 19 nM) (Gillooly et al., 2000).

Whereas FYVE fingers specifically interact with PI(3)P, PX domains generally do not exhibit the same selectivity (Lemmon, 2003). Since PI(3)P is localized to endosomes while both PI(3,4)P2 and PI(3,4,5)P3 are typically found at the plasma membrane, we next determined the intracellular localization of KIF16B *in vivo*. An affinity purified rabbit antiserum raised against recombinant, truncated (headless) KIF16B protein (see Experimental Procedures) detected the endogenous protein by confocal immunofluorescence microscopy and Western blot analysis (see below). In HeLa cells (Figure 19A), endogenous KIF16B almost completely overlapped with myc-tagged double-FYVE serving as a probe for PI(3)P on early endosomes (Gillooly et al., 2000). Given the background nuclear staining of the antibody (persisting by labelling with the pre-immune serum or upon ablation of KIF16B by RNAi, see Figure 22G), we verified that also exogenous KIF16B localized to PI(3)P-positive early endosomes (Figure 19B). No evidence for plasma membrane localization could be obtained. Importantly, at low expression levels the exogenous KIF16B motor did not significantly alter the distribution of early endosomes (see below).

In view of the binding of the KIF16B PX motif to PI(3)P *in vitro*, we next tested whether this phospholipid is required for targeting KIF16B fused to YFP (KIF16B-YFP)

to early endosomes (Gillooly et al., 2000). The generation of PI(3)P can be inhibited pharmacologically, with the fungal toxin wortmannin or LY294002 (see below). Upon treatment with 100nM wortmannin, most KIF16B-YFP detached from endosomes, leading to a diffuse cytosolic staining pattern (Figure 19C-D). EEA1, which localizes to the Rab5-domain of early endosomes in PI(3)P-dependently (Zerial and McBride, 2001), served as a positive control.

On early endosomes, Rab5 recruits effectors bearing the FYVE domain via a dual mechanisms, i.e. 1) by direct binding and 2) through generation of PI(3)P via interaction with the PI3-K hVPS34/p150 (Zerial and McBride, 2001). While we were unable to detect specific binding of KIF16B to Rab5 *in vitro* (data not shown), expression of dominant-negative Rab5S34N caused the release of KIF16B from endosomes *in vivo* (Figure 19E-F). This suggests that the membrane localization of KIF16B most likely depends on Rab5-mediated PI(3)P production. Altogether, the above experiments provide strong evidence that KIF16B is localized to PI(3)P-positive early endosomes in a Rab5- and PI3-K-dependent manner *in vivo*.

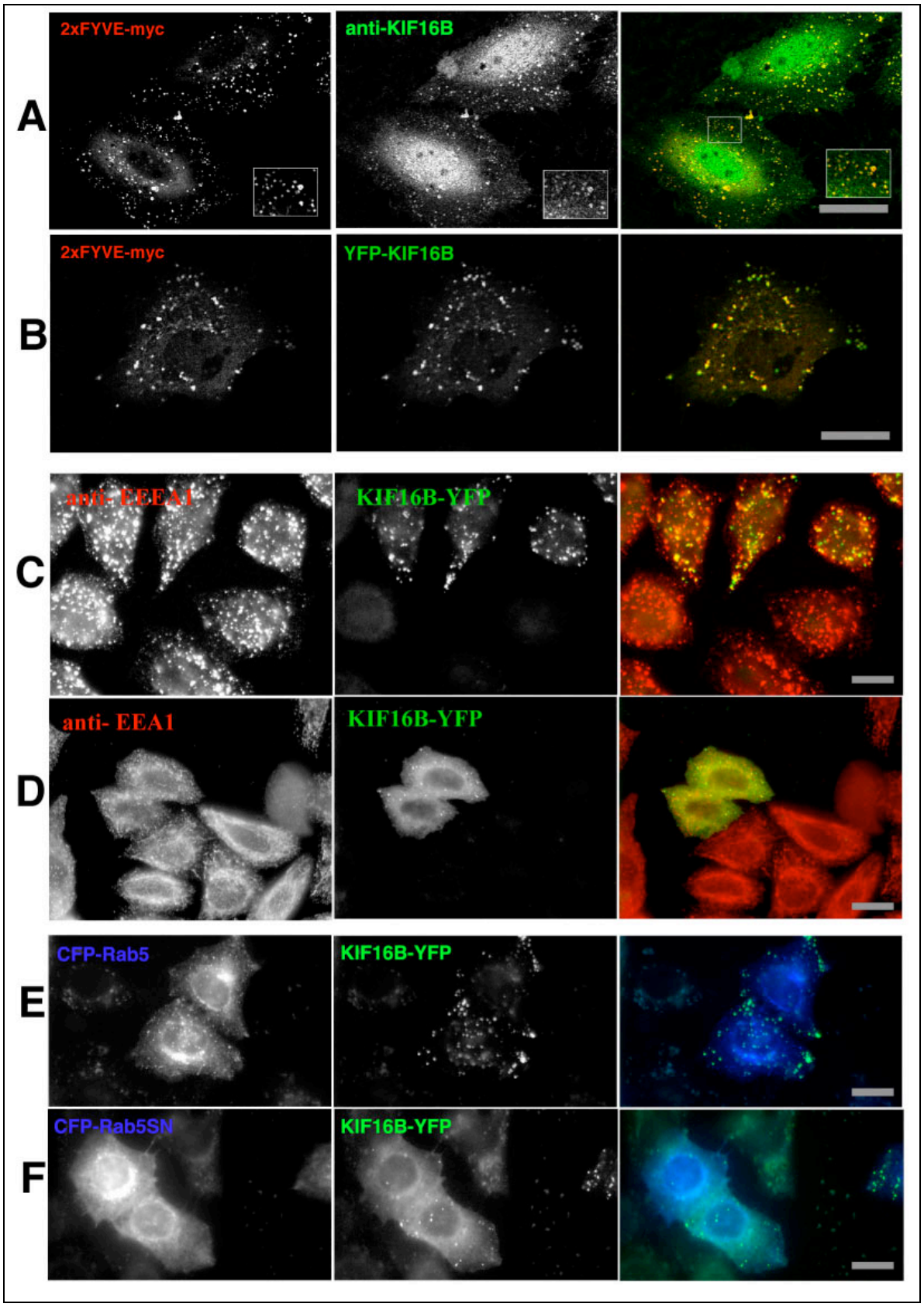

A
2xFYVE-myc
anti-KIF16B
B
2xFYVE-myc
YFP-KIF16B
C
anti- EEEA1
KIF16B-YFP
D
anti- EEA1
KIF16B-YFP
E
CFP-Rab5
KIF16B-YFP
F
CFP-Rab5SN
KIF16B-YFP

Figure 19. The kinesin KIF16B requires the presence of PI(3)P as well as Rab5 activity to localize to endosomes. *HeLa cells were transfected with the PI(3)P lipid probe 2xFYVE-myc as well as with KIF16B-YFP prior to immunofluorescence microscopy with anti-myc and anti-Kif16B antisera. The confocal images show almost complete co-localization of endogenous (A) and ectopic (B) KIF16B with the endosomal marker 2xFYVE. (C) To examine the interaction of KIF16B and PI(3)P in vivo, transfected cells expressing KIF16B-YFP were incubated in the presence (D) or absence (C) of 100nM Wortmannin for 20min at 37C and then processed for immunofluorescence for EEA1. (E) HeLa cells were co-transfected with KIF16B-YFP (green) and Rab5-CFP (blue) or KIF16B-YFP and dominant-negative Rab5-S34N-CFP (F). Overexpression of the Rab5 mutant results in release of KIF16B to the cytosol. Scale bars represent 20μm.*

VI. III. 4. Purified KIF16B moves PI(3)P-containing liposomes along microtubules *in vitro*

Having established that the PX motif of KIF16B binds PI(3)P *in vitro* and localizes to membranes bearing this lipid *in vivo*, we next tested whether recombinant full-length KIF16B, can be recruited onto PI(3)P-containing liposomes in a manner competent for transport. KIF16B was incubated with fluorescent liposomes with or without 2mol% of PI(3)P. The suspension was applied to a chamber containing fluorescently-labelled taxol-stabilized microtubules immobilized on the glass surface (Nielsen et al., 1999). True motility events, scored as movement of liposomes along the fluorescently labelled microtubule tracks, were differentiated from Brownian motion of immobile, microtubule-bound liposomes. Whereas PI(3)P-free liposomes (Fig. 20A,C) were unable to bind to microtubules (green) and diffused in the buffer in the presence of KIF16B, the PI(3)P-containing liposomes (Fig. 20B,D) were recruited to, and transported along, microtubules, exhibiting continuous motion (displacement>3μm) and velocity similar to microtubule gliding (Fig. 18). These data provide further evidence that interaction of KIF16B with PI(3)P is sufficient to move liposomes *in vitro*.

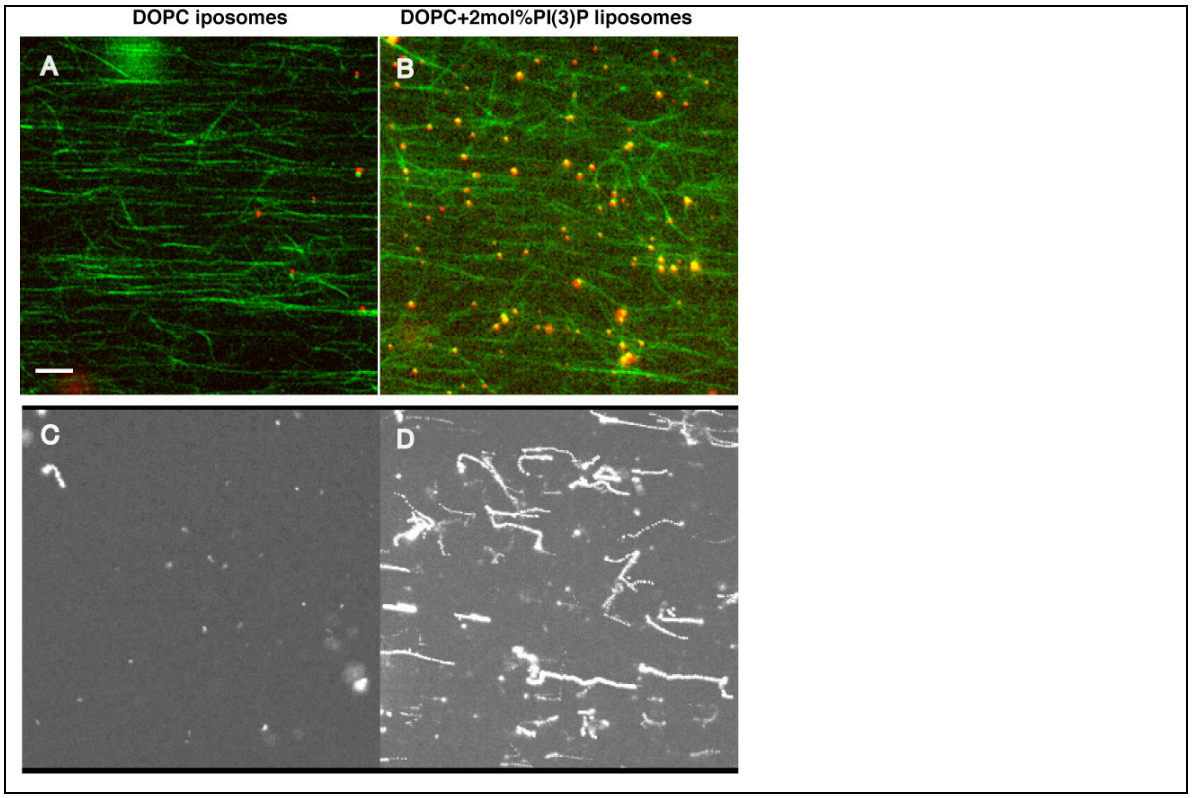


Figure 20. KIF16B confers motility to PI(3)P-containing liposomes in vitro. *The binding of KIF16B to, and movement of, liposomes in an in vitro motility assay depends on the presence of PI(3)P. Liposomes were composed of phosphatidylcholine and fluorescently labelled phosphatidylethanolamine, as a negative control (left panel), or the same mixture supplemented with 2 mol% of PI(3)P (right panel). Shown are still images of liposomes (red) and microtubules (green) in the top panel and collapsed video stacks displaying liposome movement in the bottom panel. PI(3)P-containing liposomes show motility along microtubules. Each collapsed movie represents 60 frames in a time resolution of 1 frame per 2 seconds. Scale bar-5 μm.*

VI. III. 5. KIF16B-mediated transport of early endosomes depends on Rab5 and hVPS34 activity.

To test the hypothesis that KIF16B may participate in the Rab5- and PI(3)P-dependent early endosome motility (Nielsen et al., 1999), we first confirmed the presence of endogenous KIF16B on purified early endosomes. Endogenous KIF16B co-fractionated with Rab5 and EEA1 (Figure 21A) on an early endosomes-enriched fraction (Nielsen et al., 1999). Overexpression of KIF16B increased, whereas pre-treatment of cells with wortmannin reduced, the amount of EEA1 and KIF16B on the endosomal

fraction (Figure 21A), consistent with PI(3)P being required for their membrane recruitment (Figure 19C-D).

The co-fractionation with early endosomes prompted us to explore the activity of KIF16B in an assay that recapitulates the bi-directional movement of early endosomes in a cell-free system (Nielsen et al., 1999). In this assay, early endosomes, purified from HeLa cells fluorescently labelled by internalization of rhodamine-transferrin, are combined with cytosol, energy regenerating system and buffered anti-fade mixture, and perfused into a glass chamber coated by a uniform lawn of taxol-stabilized microtubules. Moving endosomes are analyzed by video microscopy. Since KIF16B moves toward the plus-end of microtubules (Figure 18), we modified the experimental conditions to uncouple plus- from minus-ended motility in the absence of cytosol (see Experimental Procedures). In this assay, the frequency of motility events, velocities and lengths of movements of endosomes approximated the values from the cytosol-dependent assay. The motile endosomes exhibited track-lengths and velocities similar to the PI(3)P-containing liposomes (Figure 21B,C). Addition of recombinant KIF16B stimulated the frequency of motility events (Figure 21E,F). Consistent with previous data (Nielsen et al., 1999), endosome motility was Rab5- and PI(3)P-dependent. First, extraction of Rab proteins from the endosome membrane with excess of RabGDI decreased, whereas addition of Rab5-GDI complex stimulated, the number of motility events (Figure 21D,F). Second, inhibition of PI(3)-kinase either by LY294002 or specific hVPS34 function-blocking antibodies (Nielsen et al., 1999), inhibited the motility of early endosomes (Figure 21D). Importantly, endosome motility along microtubules was not only stimulated by, but also dependent on, KIF16B activity. Movement was strongly inhibited by addition of either KIF16B-ΔN, a KIF16B deletion mutant protein lacking the catalytic domain, or affinity purified anti-KIF16B antibodies. The antibody inhibition was specific as it could be overcome by supplying recombinant KIF16B protein (Figure 21E). Furthermore, in the presence of exogenous KIF16B, the system maintained its sensitivity towards Rab-GDI and Rab5-GDI complex (compare Figure 21F with 21D).

The finding that dominant-negative Rab5S34N displaces KIF16B from endosomes *in vivo* (Figure 20F), together with the dependence on Rab5 as well as hVPS34 activity

for the motility *in vitro* suggest that KIF16B is a motor protein that fulfils the biochemical properties previously established for the plus-ended motility of early endosomes *in vitro* (Nielsen et al., 1999).

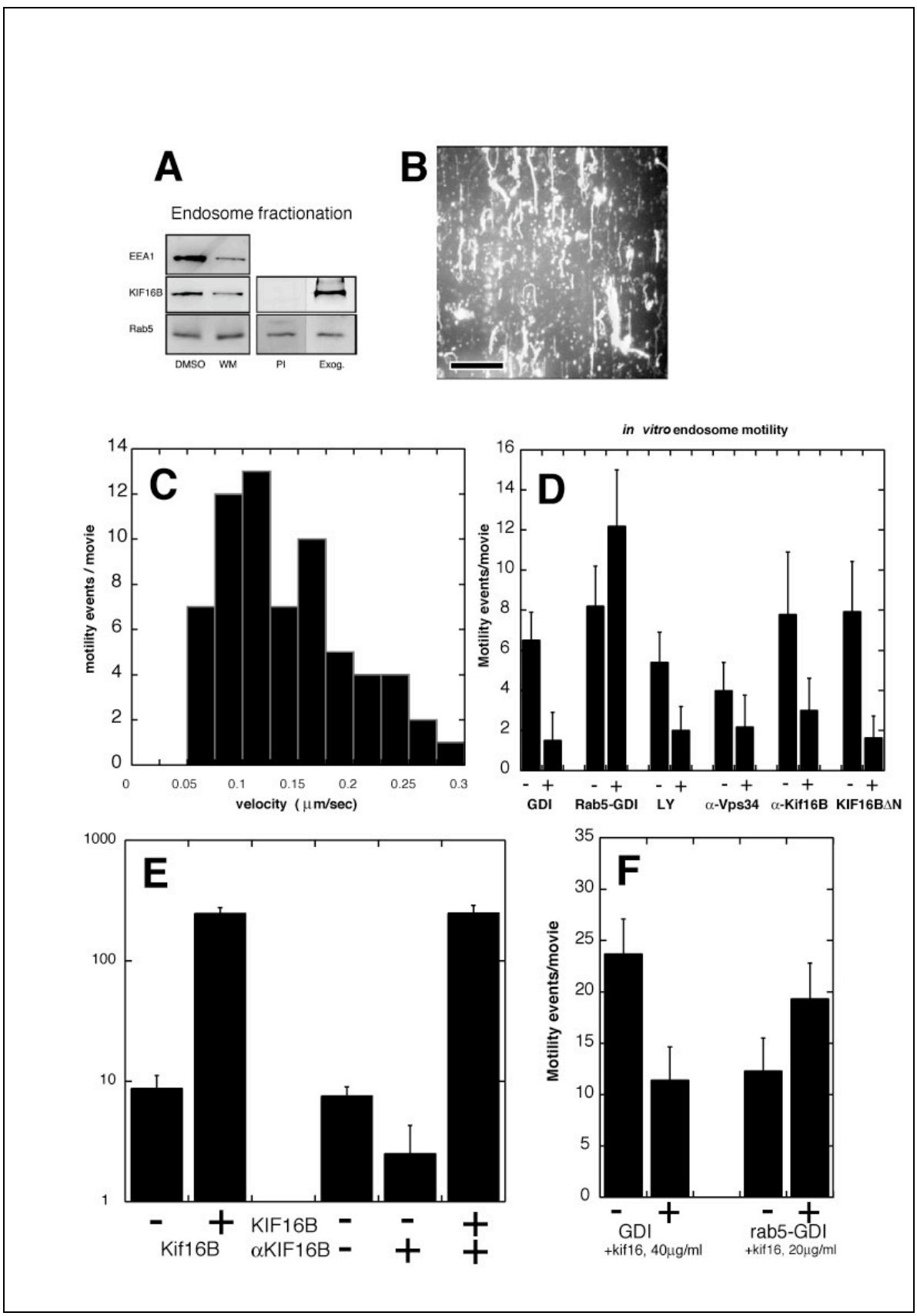

A
Endosome fractionation
EEA1
KIF16B
Rab5
DMSO
WM
PI
Exog.
B
C
motility events / movie
velocity (μm/sec)
D
in vitro endosome motility
Motility events/movie
GDI
Rab5-GDI
LY
α-Vps34
α-Kif16B
KIF16BΔN
E
Kif16B
αKIF16B
F
Motility events/movie
GDI
+kif16, 40μg/ml
rab5-GDI
+kif16, 20μg/ml

Figure 21. KIF16B co-fractionates with early endosomes and modulates plus-end motility of early endosomes in a Rab-5 dependent manner.
(A) Early endosome-enriched fractions were analysed by immuno-blotting with EEA1, Rab5 and KIF16B antibodies. Endosomes purified from cells pre-treated for 20min with 100nM Wortmannin (WM) show reduced amounts of EEA1 and KIF16B in comparison with endosomes from control cells (DMSO). Cells overexpressing exogenous KIF16B (Exog.) show an increased amount of KIF16B while KIF16B pre-immune serum shows no detectable signal on early endosomes (B) Rh-Transferin labelled early endosomes move on microtubules in the presence of KIF16B. Shown are collapsed movie images from the in vitro motility assay (see experimental procedure). An image of a collapsed movie stack displays moving endosomes as continuous lines. (C) The velocity distribution of moving endosomes in (B) shows motility events ranging from 0.05 to 0.3μm/sec. (D-F) The in vitro motility assay was carried out in the presence or absence (i.e. buffer or pre-immune serum) of various reagents and the recorded events per unit time were quantified (data are represented as mean +/- SD). Inhibition of motility is seen in the presence of RabGDI, LY2344, truncated KIF16B protein and antibodies against hVPS34 or KIF16B while a stimulation of motility was observed upon addition of recombinant KIF16B or Rab5-GDI complex. The effect of the function blocking anti-KIF16B antibodies could be rescued by excess KIF16B protein (E). In the presence of exogenous KIF16B, early endosomes retained their responsiveness to the modulation of motility by Rab5 (F). Scale bar equals 5μm.

VI. III. 6. KIF16B regulates the steady-state distribution of early endosomes

Having established a role of KIF16B in early endosome motility *in vitro*, we explored its function with respect to endosome distribution and transport *in vivo*. To this end, we compared the effects of full-length KIF16B-YFP overexpression in HeLa cells with silencing of the endogenous motor by RNA interference and the expression of two dominant-negative KIF16B mutants. The first one lacked the catalytic domain, retaining the stalk and PX domain unaltered (KIF16B-ΔN-YFP). Analogous truncation mutants have been reported to interfere with cargo localization (Noda et al., 2001). The second (KIF16B-S109A-YFP) had a point mutation in the nucleotide-binding motif, thus impairing motor activity as shown for kinesin (Nakata and Hirokawa, 1995).

Opposite effects on the intracellular distribution of early endosomes were observed in these gain- and loss-of-function studies. While early endosomes strikingly relocalized to the cell periphery upon overexpression of KIF16B-YFP (Figure 22A), either of the two KIF16B mutants (Figure 22B,C) caused their clustering in the

perinuclear region. A similar effect was produced by reducing KIF16B protein expression by ~90% using esiRNA (see Figure 24) (Figure 22E,F). Importantly, none of the various constructs resulted in any gross morphological alteration of the microtubule network, e.g. microtubule bundling or depolymerization (not shown). These results suggest that, in regulating plus-end motility, KIF16B governs the spatial distribution of early endosomes *in vivo*.

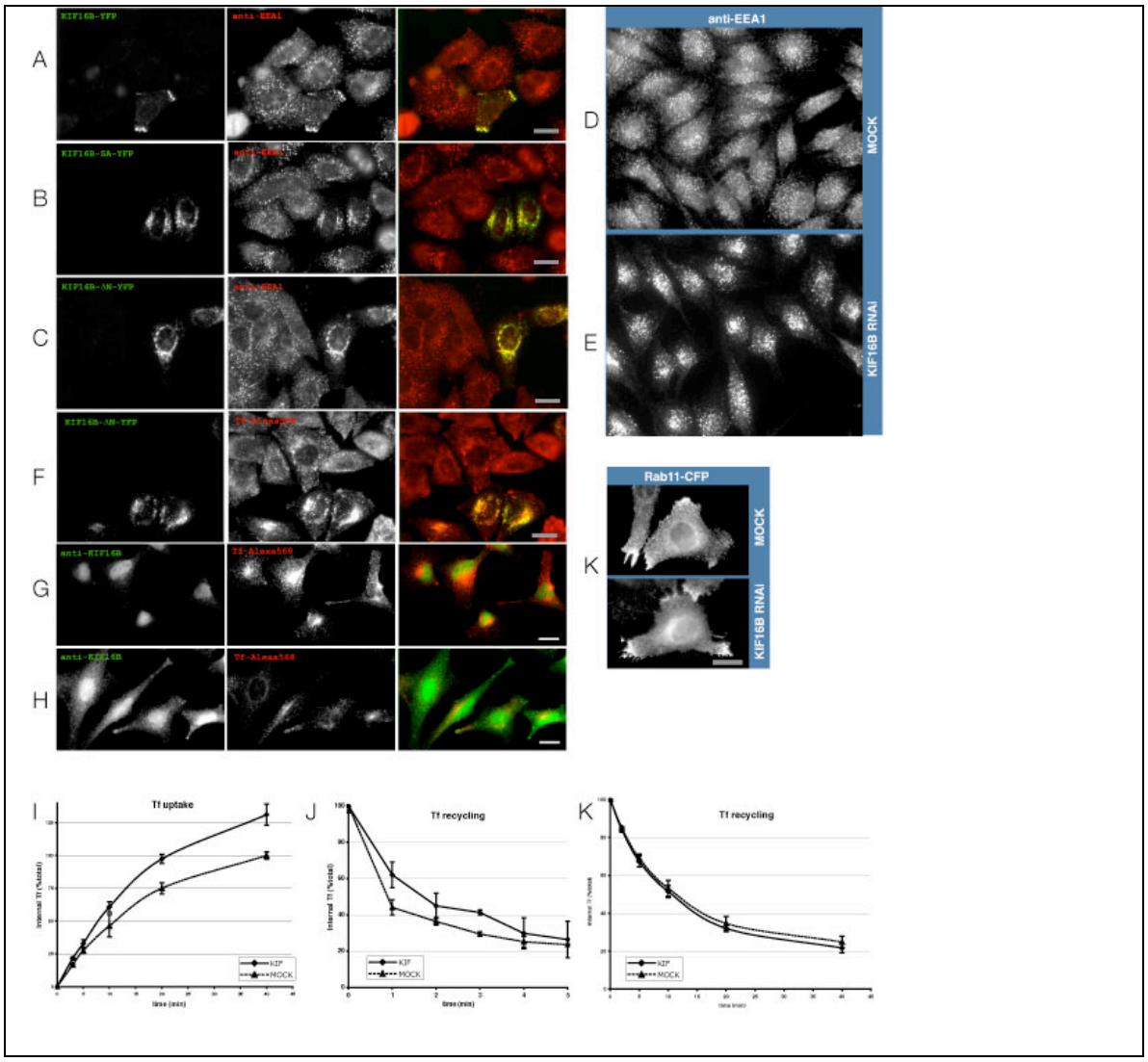


Figure 22. KIF16B regulates the steady-state distribution of early endosomes and is required for efficient transferrin recycling. *HeLa cells were transfected with KIF16B-YFP (A, green), KIF16B-ΔN-YFP (B, F; green) or KIF16B-S109A-YFP (C). 24h after transfection, cells were immunostained with antibodies against EEA1 (A-C; red) or pulsed with transferrin-Alexa568 for 30min at 37C followed by a 10min chase to remove surface bound transferrin (F; red). While EEA1 and transferrin labelled endosomes*

translocate to the cell periphery upon overexpression of wildtype KIF16B (A), they cluster in the peri-nuclear region upon expression of the dominant negative constructs (B,C,F). Also knock-down of KIF16B results in centripetal translocation of early endosomes (D,E), while the morphology of Rab11-CFP labelled recycling endosomes remains unaltered (K). (G, H, I) Tfn uptake and recycling experiments. RNAi-treated HeLa cells were allowed to take up Alexa568-labeled Tfn for the indicated times and processed for fluorescence microscopy. Ablation of endogenous KIF16B (G) leads to an intracellular accumulation of Tfn (40 minutes) in comparison with mock-treated (H) cells. (I) Cells treated with unspecific (triangles) or KIF16B-specific esiRNA (squares) were allowed to take up biotinylated Tfn for the indicated times, washed, lysed and internalized Tfn was quantified. To examine if KIF16B function affects Tfn recycling from early endosomes (J) or from recycling endosomes (K), RNAi-treated cells were subjected to pulse chase experiments with biotinylated Tfn. Cells were loaded with Tfn for 2 minutes (J) or 1h (K), washed and incubated at 37°C in medium containing 100-fold excess unlabeled Tfn. Internalized Tfn was quantified (see methods) and expressed as a percentage of total. Data from Tfn uptake and recycling assays are represented as mean +/- SD. Scale bar equals 20μm.

VI. III. 7.Ablation of KIF16B expression perturbs the recycling function of early endosomes

We next explored the role of KIF16B in the uptake of cargo into early endosomes, recycling to the surface and delivery to late endocytic compartments. We first investigated whether the activity of KIF16B affects the transferrin (Tfn) cycle. Whereas overexpression of various KIF16B constructs did not impair uptake of Alexa568-labeled Tfn (Figure 22F, not shown), ablation of KIF16B by RNAi accelerated its accumulation compared with mock-treated cells (Figure 22G,H). To quantify this effect, serum-starved HeLa cells were allowed to internalize biotinylated Tfn for various periods of time, washed, lysed and the intracellular fraction of Tfn was determined. Silencing of KIF16B indeed resulted in a faster rate of Tfn accumulation compared with control cells (Figure 22I). Since this effect could be due to a diminished recycling from early endosomes to the plasma membrane, we measured the kinetics of Tfn recycling, taking care not to chill cells to 4°C to avoid depolymerization of microtubules (Jin and Snider, 1993). Firstly, biotinylated Tfn was internalized into cells for 2 min. Under these conditions, endocytosed Tfn primarily reaches early endosomes but not perinuclear recycling endosomes. After a brief washing step to remove surface-bound Tfn, cells were chased with 100-fold excess of unlabeled Tfn for different periods of time and the amount of

biotinylated Tfn retained in the cells was quantified. Silencing of KIF16B expression delayed Tfn recycling back to the plasma membrane in comparison to mock-treated cells (Figure 22H). Secondly, when the tracer was internalized for 1 hr to saturate perinuclear recycling endosomes, no inhibition of Tfn recycling was observed (compare Fig 22J and 6K), arguing that transport from recycling endosomes to the surface was not affected. These data suggest that, besides determining the distribution of early endosomes (Fig. 21), KIF16B is also required for their efficient recycling of cargo to the cell surface.

VI. III. 8. KIF16B overexpression or ablation alters EGF trafficking *in vivo*

Transport of cargo from early to late endosomes is microtubule-dependent and requires the minus-end motor Dynein (Aniento et al., 1993). To investigate whether the accumulation of early endosomes underneath the cell cortex induced by overexpression of KIF16B impairs the sorting of cargo from early endosomes to the degradative pathway, we measured epidermal growth factor (EGF) induced receptor internalization and degradation. HeLa cells expressing either wild type or mutant KIF16B-S109A, were stimulated with EGF for 30 min or 6 hrs in the presence of cyclohexamide, to prevent de-novo synthesis of EGFR. Cells were subsequently processed for immunofluorescence staining with anti-EGFR antibodies, imaged by confocal microscopy and signal intensities quantified as described (Bache et al., 2003). In control cells, consistent with previous reports (Sorkin et al., 1991), EGFR was found in early and late endosomes during the first 30 minutes of EGF stimulation followed by almost complete degradation after 6 hours (Figure 23A). Overexpression of KIF16B but not KIF16B-S109A prevented EGF (not shown) and EGFR from entering the degradative pathway. Strikingly, EGFR persisted in early endosomes at the cell cortex over 6 hours after internalization (Figure 23A). Under these conditions, the perinuclear localization of late endosomes, as visualized by LAMP1 staining, was unaffected (Figure 23A). That EGFR accumulated in early endosomes was indicated by the colocalization with Rab5, Tfn, EEA1 and PI(3)P but not the late endosome marker LAMP1 or lysotracker DND-99 (figure 19, figure 23 and figure 24). All degradation kinetics of EGFR determined morphologically could also be confirmed biochemically (Figure 23B,C). Ablation of KIF16B by RNAi produced the

opposite phenotype, i.e. accelerated the degradation of EGFR (Fig. 23D). From these results we conclude that KIF16B regulates the transport of cargo from early to late endosomes and, consequently, its degradation.

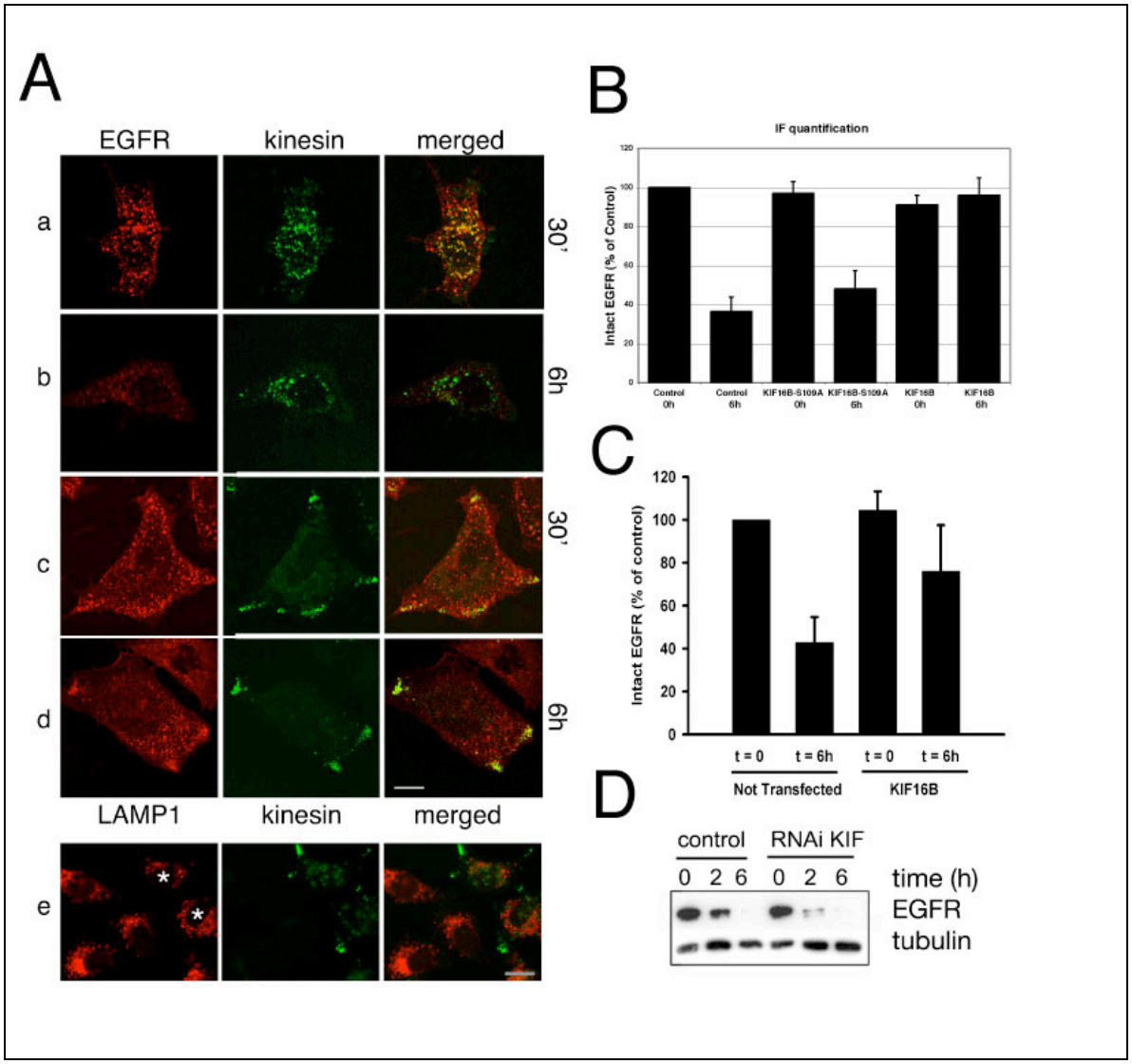


Figure 23. KIF16B modulates the degradation of endocytosed EGFR *(A) HeLa cells were transfected with full-length KIF16B-YFP or dominant-negative KIF16-S109A-YFP constructs. 24h post-transfection the cells were induced with EGF (0.1 μM) in the presence of cycloheximide (10 μg/ml) for the indicated times and immunostained with antibodies to EGFR (a-d) or LAMP1 (e). Merge shows the superimposed confocal images of the kinesin (green) and EGFR or LAMP1 (red). Mock transfected (not shown) as well as cells expressing KIF16-S109A-YFP almost completely degrade EGFR over the course of 6h (a,b). In contrast, overexpression of wild-type KIF16B-YFP inhibits EGFR degradation (c,d) while it does not affect localization of late endosomes stained with LAMP1 antibodies (e). The asterisks (*) mark the cell that overexpresses KIF16B. (B) Quantification of EGFR signal (mean +/- SEM) present in (A). (C) Cells overexpressing KIF16B show significantly less degradation of EGFR. Cells were induced for the*

indicated times with EGF, immunoblotted for EGFR and the blots were quantified. The amount of EGFR degraded is expressed as a percentage of total EGFR present before induction. The mean and SD from 3 independent experiments are shown. (D) HeLa cells were induced for the indicated times with EGF and immunoblotted for EGFR and α-tubulin as a reference. Cells pre-treated with RNAi specific for KIF16B show slightly accelerated degradation of EGFR as evident 2 hours after induction. Scale bar equals 20μm.

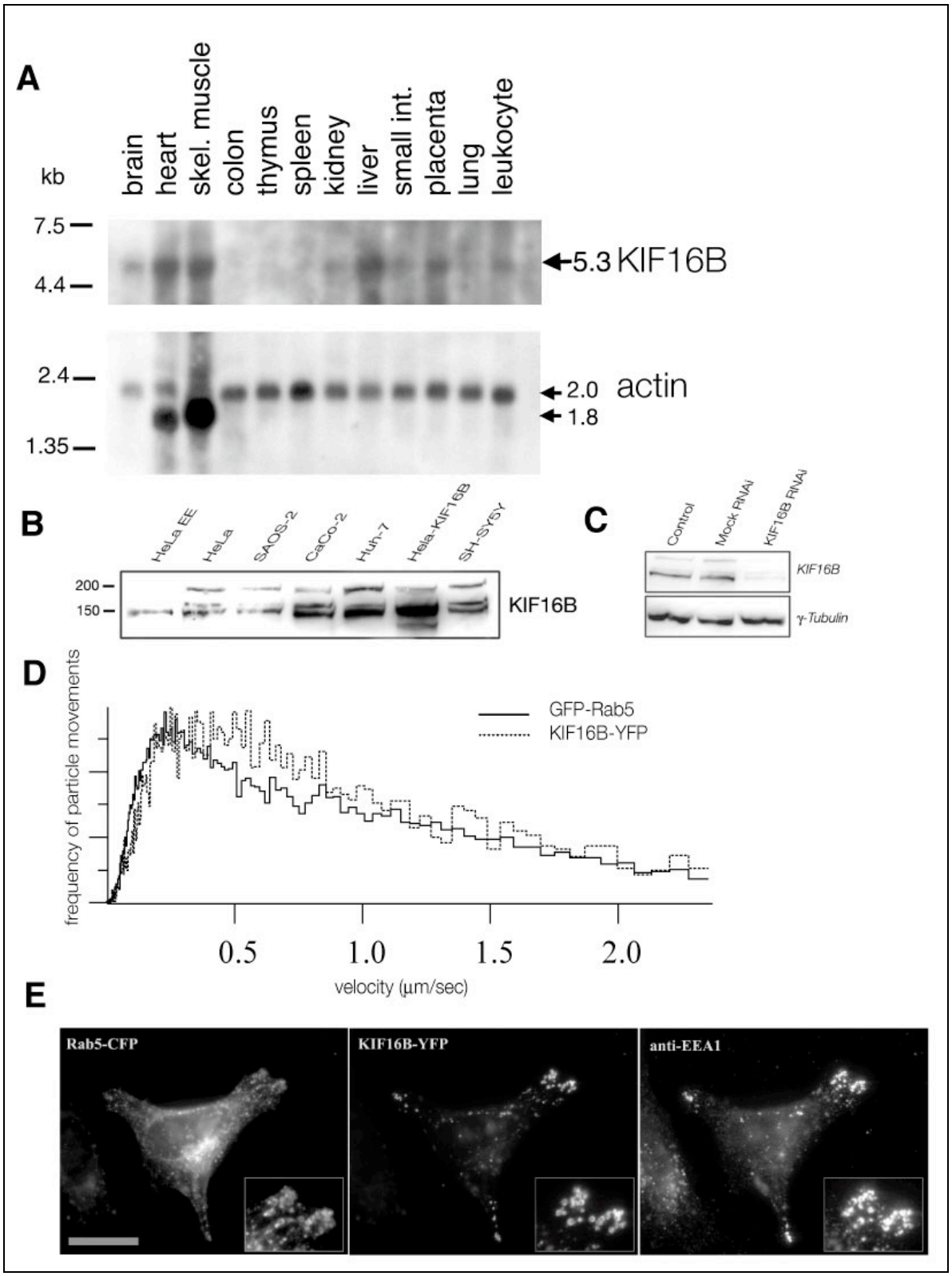


Figure 24. KIF16B is ubiquitously expressed and in vivo velocity distribution of KIF16B positive endosomes is comparable to Rab5-labeled endosomes.

(A) KIF16B mRNA is detected by Northern blot as a single 5.3kbp band. Expression is seen in brain, kidney, liver, intestine, placenta, leukocyte, heart and skeletal muscle.
(B) KIF16B protein is detected in total extracts of various human cell lines. By Western blot, KIF16B is found in total extracts of osteosarcoma (SAOS-2), hepatoma (Huh-7), colorectal adenocarcinoma (Caco-2), neuroblastoma (SH-SY5Y) and cervix carcinoma (HeLa) cells. As a reference, fractionated early endosomes (HeLa EE) as well as extract from HeLa cells overexpressing exogenous KIF16B (HeLa-KIF16B) are also shown. The 150kDa KIF16B isoform is specific for early endosomes.
(C) RNA interference in HeLa cells shows about 80% silencing of the expression of both KIF16B isoforms (250kDa and 150kDa).
(D) Quantification of the frequency of endosome movements in vivo. Movies of Kif16B-YFP or Rab5-GFP-labeled endosomes were recorded at 3.3fps and processive endosome movements which deviate less than 60% in their trajectory over 5 consecutive image frames are analyzed for their average velocity. For comparison, the velocity distribution shows the normalized frequency of such motility events. (E) KIF16B displays significant co-localization with Rab5 and EEA1. HeLa cells were co-transfected with KIF16BYFP and Rab5-CFP and subsequently stained for EEA1. Scale bar is 20µm.

VI. IV. Discussion – KIF16B

VI. IV. 1. KIF16B is a plus-end motor of early endosomes

KIF16B fulfils the functional properties expected for an early endosome kinesin. Motility studies indicated that the dynamic properties of recombinant KIF16B *in vitro* match some of the parameters measured for early endosome movement *in vivo*. First, KIF16B can transport membrane cargo over distances exceeding 3µm, thus sustaining the long-range movement displayed by early endosomes *in vivo* (Gasman et al., 2003). The short-distance movements of early endosomes are due to the association with actin filaments promoted by the small GTPase RhoD (Gasman et al., 2003). Second, in HeLa cells, ~20% of Rab5-positive early endosomes move with velocities lower than 0.4 µm/sec, ~50% 0.4-0.8 µm/sec, and ~30% higher than 0.8 µm/sec (Gasman et al., 2003). The velocities measured *in vitro* for KIF16B thus approximated the lower velocities of Rab5-labeled endosomes *in vivo* (velocity distribution around 0,35µm/sec). Conventional KHC, which propels several types of organelles (Goldstein, 2001; Goodson et al., 1997; Hirokawa, 1998) displays ~2-3 fold higher velocities *in vitro* (Vale et al., 1985; von Massow et al., 1989). Early endosomes may well rely on multiple kinesins for propulsion, also depending on the cell type. For example, KIFC2, which was implicated in early endosomal motility, is likely restricted to neurons (Yang et al., 2001). However, beside its ubiquitous expression, a unique feature of KIF16B among KIFs is that it

contains an important hallmark of early endosome targeting, the PX domain. This domain allows KIF16B to bind and move PI(3)P-containing liposomes and localize to early endosomes. Importantly, *in vivo* studies confirmed the idea that KIF16B is required for the proper intracellular distribution of early endosomes.

Next to the well-characterized Unc104/KIF1A, KIF16B represents the only other kinesin motor with the ability to directly interact with lipids. Since PX-domains frequently require dimerization to bind PI(3)P (Lemmon, 2003), it is conceivable that the high coiled-coil potential of its stalk is sufficient for KIF16B to dimerize. This raises the question of whether the regulation of KIF16B motility follows a cooperative model as proposed for Unc104 and Kif1A, for which clustering in lipid rafts leads to high density of motor monomers, thus facilitating dimerization and processive transport (Klopfenstein et al., 2002). Given its high coiled-coil propensity, KIF16B is in all likelihood a dimer like *D. discoideum* Unc104 and does not follow this cooperative transition upon recruitment to early endosomes.

VI. IV. 2. Coupling of membrane fusion with early endosome motility via Rab5 and PI(3)P

In displaying Rab5 and PI(3)P-containing cargo selectivity, a remarkable property of KIF16B is that it is subjected to the same regulatory principles governing the membrane tethering and fusion machinery (Zerial and McBride, 2001). Even though Rab5 and KIF16B do not appear to interact directly, Rab5 can modulate the generation of PI(3)P in its vicinity via its direct interaction with hVPS34/p150, (Christoforidis et al., 1999b) (Shin et al, submitted), thus allowing for the binding of the PX domain of KIF16B to the endosomal membrane. This mechanism reiterates the role of Rab5 in creating a specialized membrane domain where the activities of different effectors can be kinetically controlled by the GTPase cycle and coordinated through spatial segregation within restricted sub-compartments of the early endosome (Zerial and McBride, 2001).

Our study strengthens the emerging paradigm that Rab GTPases, besides functioning in membrane fusion, also orchestrate organelle movement. Rab11 and Rab27 regulate the membrane recruitment of myosin motors onto melanosomes and recycling endosomes (Hales et al., 2002; Wu et al., 2002). Rabkinesin-6 was proposed to be an

effector of Rab6 regulating the movement of Golgi membranes (Echard et al., 1998), although recent studies rather argue for a function in cytokinesis (Hill et al., 2000). Finally, the Rab7 effector RILP was shown to indirectly regulate the recruitment of the Dynein-Dynactin complex to late endosomes (Jordens et al., 2001).

In the case of early endosomes, Rab5 and PI(3)P appear to regulate both the plus- and minus-end motility of early endosomes (Nielsen et al., 1999), raising the question of how bi-directional movement is regulated. For example, the bi-directional movement of melanosomes is coordinated by Dynactin which binds both Dynein and the plus end-motor kinesin II (KIF3) (Deacon et al., 2003; Valetti et al., 1999). In the absence of cytosol, isolated early endosomes exhibit only plus-end motility *in vitro*, suggesting that minus-end directed motors must either be lost or inactivated during the fractionation procedure. However, rather than the direction of movement being determined by stochastic motor recruitment or competition between motors of opposite polarity, precise regulatory mechanisms are required to switch them on and off (Reilein et al., 2001). A possible regulatory mechanism for the activity of KIF16B could be phosphorylation, given the presence of a phospho-threonine/serine-binding FHA domain (Durocher and Jackson, 2002).

VI. IV. 3.The role of KIF16B in endosome motility, cargo transport and signal transduction

Our data suggest that microtubule-dependent motility plays a role in the regulation of endocytic transport at earlier stages than previously assumed. Microtubule-dependent motility facilitates trafficking from early to late endosomes but is not required for the recycling of ligand/receptor complexes from early endosomes to the surface (Apodaca, 2001), a process that rather depends on actin (Apodaca, 2001). Our data suggest that plus-end motility along microtubules may facilitate the targeting of recycling carriers from early endosomes to the plasma membrane. This may be a prerequisite to switch from microtubule- to actin-based transport to allow efficient cargo exocytosis. Proximity of early endosomes to the cell cortex may also favour fast over slow recycling via perinuclear recycling endosomes (Maxfield and McGraw, 2004), as also observed for

Rabenosyn-5 (de Renzis et al., 2002). Interestingly, inhibitors of PI(3)-kinase cause a significant retention of transferrin in early endosomes (van Dam et al., 2002). Such inhibition could be explained, at least in part, by the release of KIF16B from the early endosome membrane (Figure 19).

Transport along the degradative pathway, either mediated by endosomal carrier vesicles (ECVs) that bud from early and fuse with late endosomes, or occurring by a maturation process (Gruenberg and Maxfield, 1995), has been shown to exclusively depend on Dynein (Aniento et al., 1993). Our findings that KIF16B modulates EGFR degradation, suggest that the activity of KIF16B balances that of Dynein in regulating the transport of cargo from early to late endosomes. We have observed that transition from early to late endosomes occurs by conversion of Rab5- into Rab7-positive structures, a process occurring near the centre of the cell (Rink et al., submitted). Based on the effects of KIF16B, we suggest that the spatial distribution of early endosomes is critical to their ability to either generate ECVs or fuse them with, or convert into late endosomes. Consistently, the peripheral endosomes induced by KIF16B overexpression harboured early but failed to acquire late endosome markers. We propose that early endosomes can acquire cargo and recycle it to the cell surface as long as the Rab5 and PI(3)P-dependent recruitment of KIF16B keeps them near the periphery. Once the activity of Dynein prevails over that of KIF16B, the endosomes predominantly reside in the centre of the cell where they become committed to switch to the degradative pathway. Interestingly, when late endosomes were relocated to the cortex of the cell by Dynamitin overexpression, transport from early to late endosomes was inhibited despite the close proximity of these organelles to each other (Valetti et al., 1999), further supporting the idea that intracellular localization governs the transport activity of these organelles.

Our results on the effect of KIF16B on EGF degradation have important implications for signal transduction. Prior to degradation, internalized receptors continue signalling by activating cytoplasmic target molecules (Miaczynska et al., 2004a). Since receptor downregulation results in signal desensitization over time, the trafficking role of KIF16B may also impact on the signalling response. Remarkably, KLP98A, the Drosophila ortholog of KIF16B has previously been linked to *wingless (wg)* signalling

(Dalby, 1996), opening the interesting avenue of exploring the role of KIF16B during development. It will also be interesting to explore the role of KIF16B in endocytic and transcytotic trafficking in polarized cells. Addressing these questions will improve our understanding of the physiological role of cytoskeleton-mediated endocytic transport at the cellular and multi-cellular level.

VI. V. Outlook - KIF16B and endosome motility

As mentioned before, Rab27 and its effector myosinVa mediate actin dependent motility of melanosomes. As a consequence, in the absence of functional myosinVa or Rab27a, melanosomes are transported bi-directionally along microtubules near the center of the cell, but are unable to bind to actin in the cell periphery (Bahadoran et al., 2001; Hume et al., 2001; Wu et al., 2001). With respect to endosomes, overexpression of the Rab5-effector huntingtin-associated protein 40 (HAP40) has been shown to lock early endosomes to actin cables while preventing their switch to microtubules-dependent motility (Arun Pal, manuscript submitted). Interestingly, early endosomes which are immobilized on actin upon HAP40 overexpression do not acquire KIF16B. This raises the question, if, in analogy to Rab27 on melanosomes, the acquisition of actin-based motors precedes the recruitment of microtubules-dependent motors such as KIF16B or if early endosomes are constantly oscillating between actin- and microtubules-dependent motility.

Binding of YXXø-bearing cargo such as transferrin receptors to the AP2 adaptor complex requires phosphorylation of the AP2 μ-subunit by AAK1 (Adaptor Associated Kinase-1). As a consequence, AP2 dependent endocytosis (of transferrin receptors, for example) is severely compromised in the absence of this phosphorylation event. Concomitantly, the number of clathrin coated pits is severely reduced. Interestingly, silencing of AAK-1 function by RNAi leads to a striking accumulation of Tf positive

early endosomes in the perinuclear area (Pelkmans et al., 2005). With respect to endosome motility this leads to two possible hypothetical scenarios:

(1) since early endosomes exhibit a net movement to the center of the cell (Jochen Rink et al. manuscript submitted), downregulation of AP2 mediated endocytosis at the plasma membrane could eventually lead to the bulk delivery of all remaining endosomes to the cell center over time.

(2) The directionality of endosome motility is controlled through cargo present within endosomes. For example: in the absence of cargo that needs to be recycled to the surface such as transferrin receptors, endosomes could commit to travel to the perinuclear region within the cell where they convert into or fuse with late endosomes (J. Rink et al., manuscript submitted).

Upon overexpression of KIF16B, early endosomes migrate to the cell periphery while compromising EGF degradation. Cancer cells could exploit this mechanism to achieve increased signaling activity from growth factor receptors. Interestingly, tumors isolated from BRCA1 positive patients (n=18) exhibited a significantly reduced expression of KIF16B (van 't Veer et al., 2003). Also patients with breast tumor cells that infiltrated the lymphatic system (n=28) showed a reduced expression of KIF16B (van 't Veer et al., 2003). In this light it would prove interesting to examine:

(a) how EGFR signaling is modulated upon ablation of KIF16B.

(b) if KIF1 6B ablation leads to an increase in cancer cell motility or invasiveness.

In this context it will be also interesting to compare endosome function in healthy and diseased tissue to study if subversion of certain endocytic components such as molecular motors, for example, by cancer could directly result in aberrant signal attenuation, uncontrolled cell proliferation and neoplasia.

VI.I. Results and discussion part II: Genome-wide RNAi screen for polarised membrane trafficking in C. elegans

VI. I. 1. Choice of a system: apical transport in the *C. elegans* enterocyte.

It was in 1963, when the Nobel laureate Sydney Brenner had for the first time communicated his vision of how a tiny Nematode worm could help tackle the next challenge in molecular life sciences. Sydney Brenner outlined this next challenge in his proposal to the Medical Research Council, in October of 1963: "The new major problem in molecular biology is the genetics and biochemistry of control mechanisms in cellular development". This marked the birth of the field of c.elegans research which quickly expanded.

Research on the nematode *C. elegans* has contributed greatly to elucidate mechanisms behind key cellular functions and development. Surprisingly, this organism has been explored to a limited degree for studying intracellular transport and organelle biogenesis (Nurrish, 2002). Most importantly, C.elegans offers a possibility to study membrane traffic in polarized tissues. Being amenable to high-throughput screening, C.elegans can be utilized as a system to revisit how cargo sorting, recycling through and motility of endosomes is implemented in polarized cells. To address the questions defined in the chapter 'conceptual outlook', I found the intestine of this nematode most suitable for high-throughput screening for several reasons:

- The suitability of *C. elegans* as a model system to identify genes functioning in endocytosis has been recently demonstrated. Using assays that measure uptake of a ligand or fluid phase marker, it has been possible to identify a set of interesting new regulatory components for endocytosis and recycling (Fares and Greenwald, 2001; Grant and Hirsh, 1999).
- In contrast to approaches based on cultured cells, the intestine of the nematode offers superior morphological resolution (see also figure 11) as well as the

advantage of studying interplay between recycling and polarity in a *bona fide* tissue.

- Compared to many other organisms, C.elegans relies mainly on key components required for membrane trafficking (Bock et al., 2001), maximizing the chances to detect strong phenotypes due to minimal redundancy between components.
- C.elegans is amenable to high throughput reverse genetic screening using RNAi technology (Gonczy et al., 2001; Poulin et al., 2004)

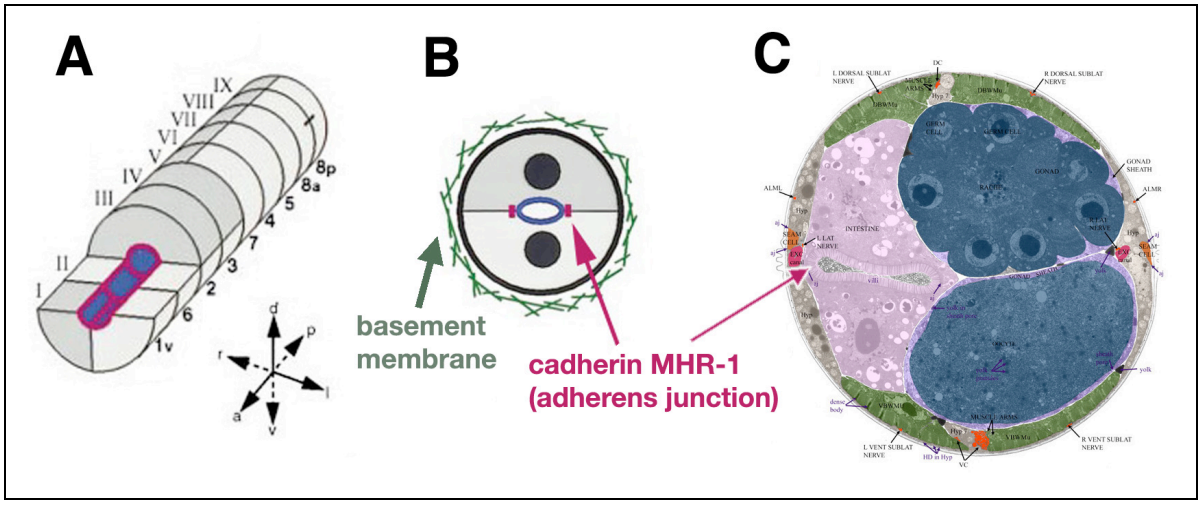


Figure 11
Architecture of the intestinal system in C.elegans.
(A and B) distribution of adherence junctions in the polarized enterocytes. The C.elegans intestine consists of 20 polarized epithelial cells. Adherens junctions are in red, the apical plasma membrane in blue. Images adapted from (Leung et al., 1999) (B) EM picture of a cross section through the intestine of C.elegans illustrating the morphology of two enterocytes facing each other. Adherence junctions ('aj') and the brush border of the apical plasma membrane ('villi') are indicated. Pictures taken from http://www.wormatlas.org.

VI. I, 2. Search for suitable markers and promoters as a platform for the visual screen

Endocytosis and exocytosis are intrinsically coupled. Depending on the marker and cellular system utilized, sorting to the apical plasma membrane can occur at the level of basolateral early endosomes (Mostov et al., 2000) or apical recycling endosomes (Ang

et al., 2004). The apical localization at steady state is predicted to be the result of a dynamic process that depends on biosynthetic trafficking to the apical surface, apical endocytosis and recycling. Deviation from the apical localization at steady state upon RNA mediated silencing, should be indicative that known as well as novel components required for apical sorting, tethering, fusion, endocytosis and recycling are affected. By following the polarized sorting and delivery of an apical marker to the plasma membrane in enterocytes of C.elegans also new molecular motors as well as polarity factors could be identified. It is not clear if apical transport occurs via a direct route from the Golgi to the plasma membrane or indirectly involving sorting at the basolateral early endosome or also the apical recycling endosome in these cells. Nevertheless, most proteins which are endocytosed apically will be recycled via the recycling endosome by default. Surprisingly little is known about how and why apical endosomes maintain their polarized localization and which components regulate transport between this endocytic compartment and the cell surface. By recording morphological changes of the apical recycling endosome upon RNAi, aberrant structure or localization of this organelle should highlight genes that govern its polarized distribution and function and might give new insights into how its subcellular structure is linked to its physiological function.

Unexpectedly, many prominent transmembrane markers suitable to study apical transport and recycling are either not conserved (such as the transferrin receptor or the IgG receptors) or they are secreted in C.elegans (such as sucroseisomaltase, for example). Furthermore, expression of both markers need to be restricted in time and space to maximize morphological resolution.

An expression screen (see table 3 and figure 12) combining multiple promoters, signal sequences and markers yielded several transgenic worms which can be utilized for the forthcoming screen and subsequent secondary screens.

Table 3: Plasmids constructs generated to create transgenic worms

No.	Promoter	SigSeq	Marker Gene	Plasmid Back-bone
1	-	integrin beta (pat-3)	-	YFPN (Andy Fire)
2	-	integrin beta (pat-3)	-	YFPN
3	-	integrin beta (pat-3)	-	CFPN (Andy Fire)
4	Vit-6	integrin beta (pat-3)	-	YFPN
5	Cpr-1	integrin beta (pat-3)	-	YFPN
6	Vit-6	integrin beta (pat-3)	T13C2.6	YFPN
7	Cpr-1	integrin beta (pat-3)	T13C2.6	YFPN
8	Vit-6	integrin beta (pat-3)	Lrp-1	YFPN
9	Cpr-1	integrin beta (pat-3)	Lrp-1	YFPN
10	Cpr-1	integrin beta (pat-3)	CE04815	YFPN
11	vit-6	integrin beta (pat-3)	CE04815	YFPN
12	Vit-6	T13C2.6	T13C2.6	YFPN

13	Vit-6	Lrp-1	Lrp-1	YFPN
14	Vit-6	Lrp-1	HA	YFPN
15A	Vit-6	T13C2.6	T13C2.6	YFPN
16B	Vit-6	Lrp-1	Lrp-1	YFPN
17A	Vit-6	T13C2.6	T13C2.6	YFPN
18A	Vit-6	Lrp-1 (91bp)	HA	YFPN
19A	Vit-6	Lrp-1 (91)	HA	YFPN
20AB	Vit-6	Lrp-1 (91)	Lrp-1 (915)	YFPN
21A	Vit6	-	CeRab11	YFPN
22	Vit6	Lrp1 (2.3k)	-	YFPN
23B	Vit6	T13C2.6 (500)	-	YFPN
24	Vit6	T13C2.6 (500)	Lrp-1 (1251)	YFPN
25	Vit6	Lrp1 (2.3k)	Lrp-1 (1251)	YFPN
26	Cpr1	-	Rab11	YFPN
27	Cpr1	Lrp-1 (2316bp)	HA	YPFN
28	Cpr1	Lrp-1 (2316bp)	Pho-1	YFPN
29	Vit6	T13 (500)	Pho-1	YFPN
30	Vit6	Lrp-1 (2316bp)	Pho-1	YFPN
31	Vit6	Lrp-1 (2316bp)	Asp-1	YFPN
32A	Cpr1	Lrp-1 (91bp)	Nhx-2 (980) G>E pointmut.	YFPN
32B	Cpr1	Lrp-1 (91bp)	Nhx-2 (980)	YFPN
33	Cpr1	MCS	MCS	YFPN
34	Ges1	MCS	MCS	YFPN
35	Vit6	Lrp-1(91)	Nhx-2 (980)	YFPN
36	Cpr-1	--	Rab11; unc-119	YFPN-Unc119
37	Vit-6	--	Rab11; unc-119	YFPN-Unc119
38	Vit-6	Lrp-1 (91bp)	Tre	YFPN
39	Vit-6	MCS	MCS	YFPN
40	Vit-6	MCS	Rab5	YFPN
41	MCS	MCS	Rab5	CFPN
42	Vit-6	MCS	Rab5	CFPN
43	Vit-6	MCS	Rab5	CFPN-Unc119
44	Vit-6	Lrp-1 (91bp)	Rab5	CFPN-Unc119
45	Vit-6	Lrp-1 (91bp)	PHO-1	CFPN-Unc119
46	Vit-6	Lrp-1 (91bp)	Asp-1	CFPN-Unc119
47	Vit6	Lrp1 (91)	Nhx2 (980)	CFPN-Unc119
48	Vit6	Lrp1	Tre	CFPN-Unc119
49	Ges1	MCS	Rab11	YFPN-Unc119
50	Vit6	MCS	Rab5	YFPN-Unc119
51	Vit6	Lrp-1(91bp)	Rab5	YFPN-Unc119
52	Vit6	Lrp-1(91)	HA	CFPN-Unc119
53	Vit6	MCS	Rab5QL	YFPN-Unc119
54	Ges-1	MCS	MCS	YFPC-Unc119
55	Ges-1	MCS	MCS	CFPC-Unc119
56	Pie1	GFP	Unc50	PAZ-Unc119
57	Ges-1	--	Asp1	YFPC-Unc119
58	Ges-1	--	Asp1	CFPC-Unc119
59	Ges-1	--	Pho1	YFPC-Unc119
60	Ges-1	--	Pho1	CFPC-Unc119
61	Ges-1	--	Nhx2	YFPC-Unc119
62	Ges-1	--	Nhx2	CFPC-Unc119
63	Cpr-1	--	Rab5	YFPN-Unc119
64	Sip1	--	--	YFPC-Unc119
65	Ges-1	--	Rab5	YFPN-Unc119
66	Ges-1	--	Rab5-Q79L	YFPN-Unc119
67	Vit6	Lrp-1(91bp)	MCS	CFPN-Unc119
68	Vit6	Lrp-1(91bp)	MCS	YFPN-Unc119
69	Ges-1	MCS	Nhx(980 bp)	CFPC-Unc119
70	Ges-1	MCS	Nhx(full)	YFPC-Unc119
71	Vit-6	Lrp-1 (91)	Nhx(980 bp)	YFPN
73	Pie1	GFP	Unc50	PAZ-Unc119
74	Vit6	Lrp-1(91bp)	LRP-1(918)	YFPN-Unc119
75	Vit6	Lrp-1(91bp)	LRP-1(918)	CFPN-Unc119
76	Ges-1	Lrp-1(91bp)	MCS	YFPN-Unc119
77	Ges-1	Lrp-1(91bp)	MCS	CFPN-Unc119
78	Ges-1	Lrp-1(91bp)	MCS	YFPC-Unc119
79	Vit-6	--	Nhx2	CFPC-Unc119
84	OPT-2	--	OPT-2	GFP-Unc119
85	Nhx-2	--	Nhx-2	GFP-Unc119
86	Ges-1	Lrp(91)	Tre(CT)	CFPN-Unc119
87	OPT-2	--	OPT-2	CFPN-Unc119
88	OPT-2	--	OPT-2	YFPN-Unc119
89	OPT-2	--	MCS	CFPN-Unc119
90	OPT-2	--	MCS	YFPN-Unc119
91	OPT-2	--	OPT-2	DsRED2-Unc119
92	Ges-1	-	Rab11	GFP-Unc119
93	Vit-6	-	Rab11	GFP-Unc119
94	Opt-2	-	Rab11	YFPN-Unc119
95	Opt-2	-	Rab5	YFPN-Unc119
96	Opt-2	-	Rab11	GFP-Unc119

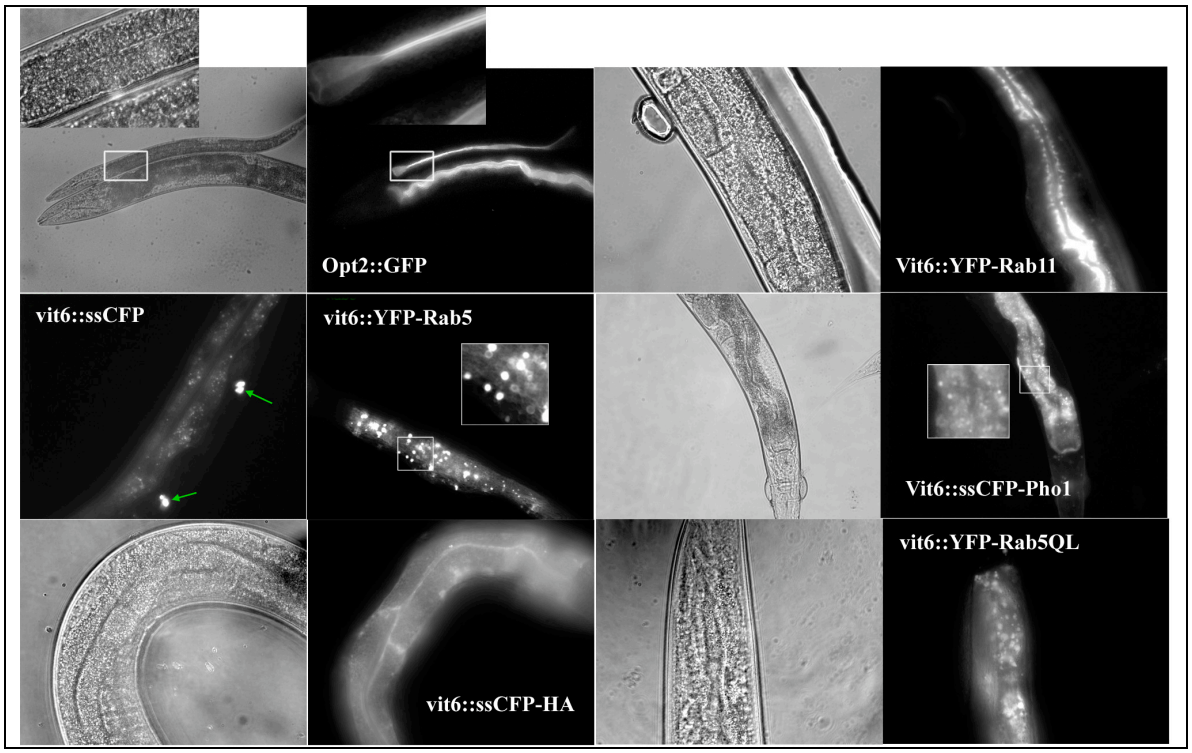


Figure 12:
Selected set of C.elegans strains expressing various marker transgenes.
The multispan transmembrane peptide transporter Opt-2 and the small GTPase Rab11 both exhibit a polarized distribution within the enterocytes at steady state (top). Secreted CFP (vit6::ssCFP; middle) is taken up by fluid phase endocytosis into coelomocytes (arrows). This strain and the Rab5 and Rab5QL markers can be utilized for secondary assays. Transmembrane proteins which are either integrated into both the apical and basolateral membrane (ssCFP-HA) or remain entrapped in intracellular vesicles (ssCFP-Pho1) might prove valuable to study transport to the basolateral membrane or fusion events between endomembrane compartments respectively.

VI. I, 3. Subcellular distribution and morphology of the recycling endosome

The morphology of the subapical recycling endosome (ARE) is strongly dependent on expression levels of the small GTPase Rab11 (see Figure 13). Furthermore, the morphology of Rab11 positive apical recycling endosomes is altered upon co-expression of the Opt-2 marker or by treatment of worms with brefeldin A (BFA) (figure 14). BFA induces recycling endosome tabulation (Lippincott-Schwartz et al., 1991; Sonnichsen et al., 2000), possibly by interfering with ARF1, which is also conserved in C.elegans (Bock et al., 2001), confirming the identity of the Rab11 positive compartment

in C.elegans. It is not clear, why co-expression of Opt-2 and Rab11 induces a morphological alteration of the recycling endosome, since at steady state no Opt-2 localizes to the apical recycling endosomes (see figure 14). One possibility could be that excess Opt-2 at the apical plasma membrane changes the rate of bulk-endocytosis which consequently might also affect cargo flow through the apical recycling endosome. The function of the RME-1 gene has been linked to the function and distribution of recycling endosomes (Lin et al., 2001). Interestingly, RNAi mediated depletion of this protein generates large vacuoles at the basolateral side of C.elegans enterocytes but it does not significantly disturb the apical positioning of Rab11 positive endosomes (see figure 14 bottom panel). Furthermore, these vacuoles are Rab11 negative, questioning the direct functional link between RME-1 and the canonical Rab11-positive recycling endosome. RME-1 might be required for recycling to the basolateral side perhaps involving another Rab GTPase possibly gum-1 (Chih-Hsiung Chen, Peter Schweinsberg, Eric Lambie, Barth Grant, unpublished observation).

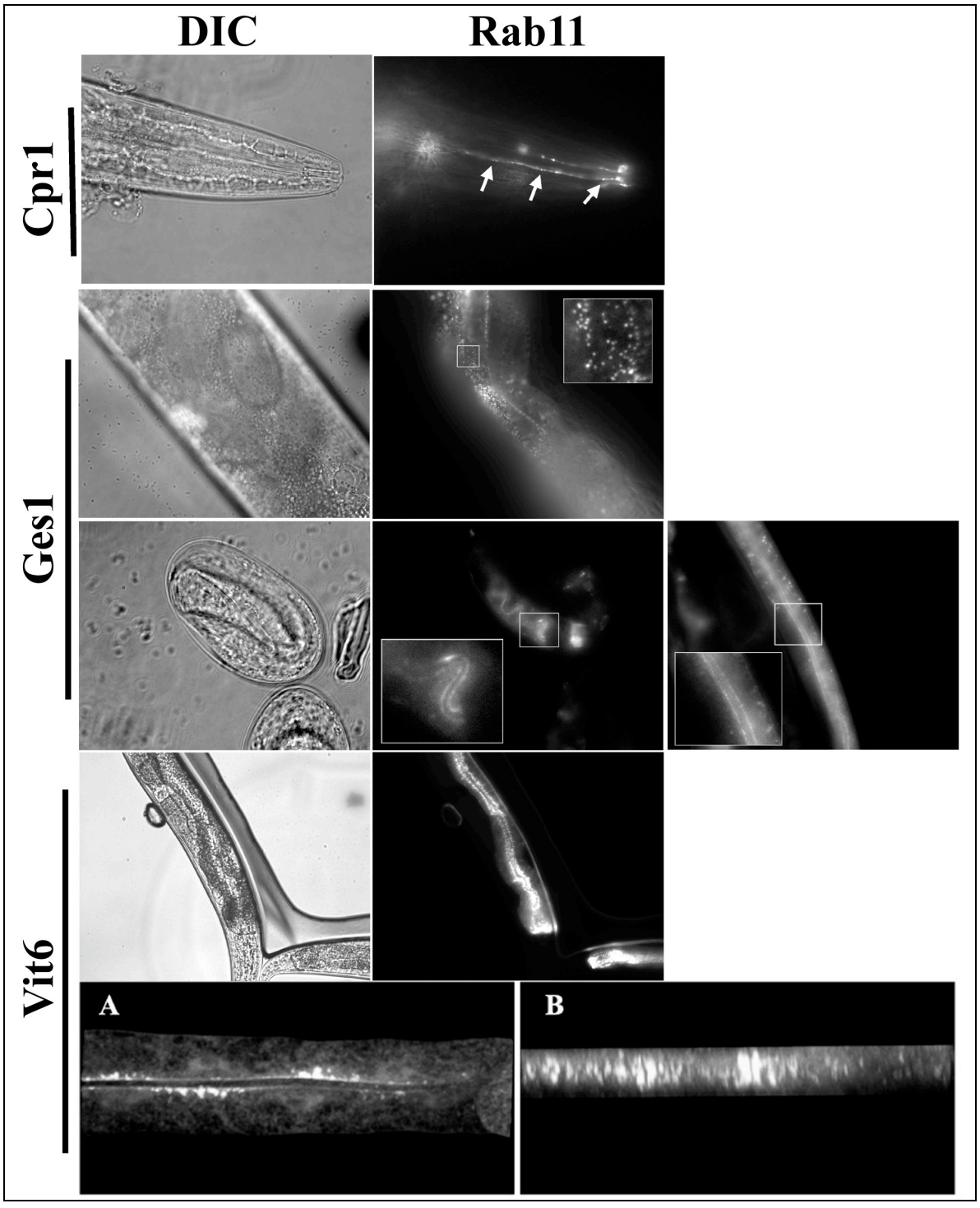


Figure 13
Subapical recycling endosomes exhibit bi-directional motility and change morphology depending on expression level.
Expression under a truncated CPR-1 promoter labels Rab11 positive endosomes in phasmid neurons (top) which exhibit bi-directional motility. The moderately strong promoter of gut-esterase-1 (Ges-1) specifically expresses Rab11 in enterocytes of the

developing embryo, larval stages, and adult worms. Under this promoter, Rab11 endosomes are relatively small, segregated, dotted in appearance and proximal to the apical plasma membrane. At elevated expression levels (Vit6 promoter), a 2-photon 3D reconstruction (bottom panel) reveals Rab11 positive recycling endosomes residing underneath the apical membrane. However, the endosomes now appear more clustered (a 90-degree rotated intestine is presented on the right).

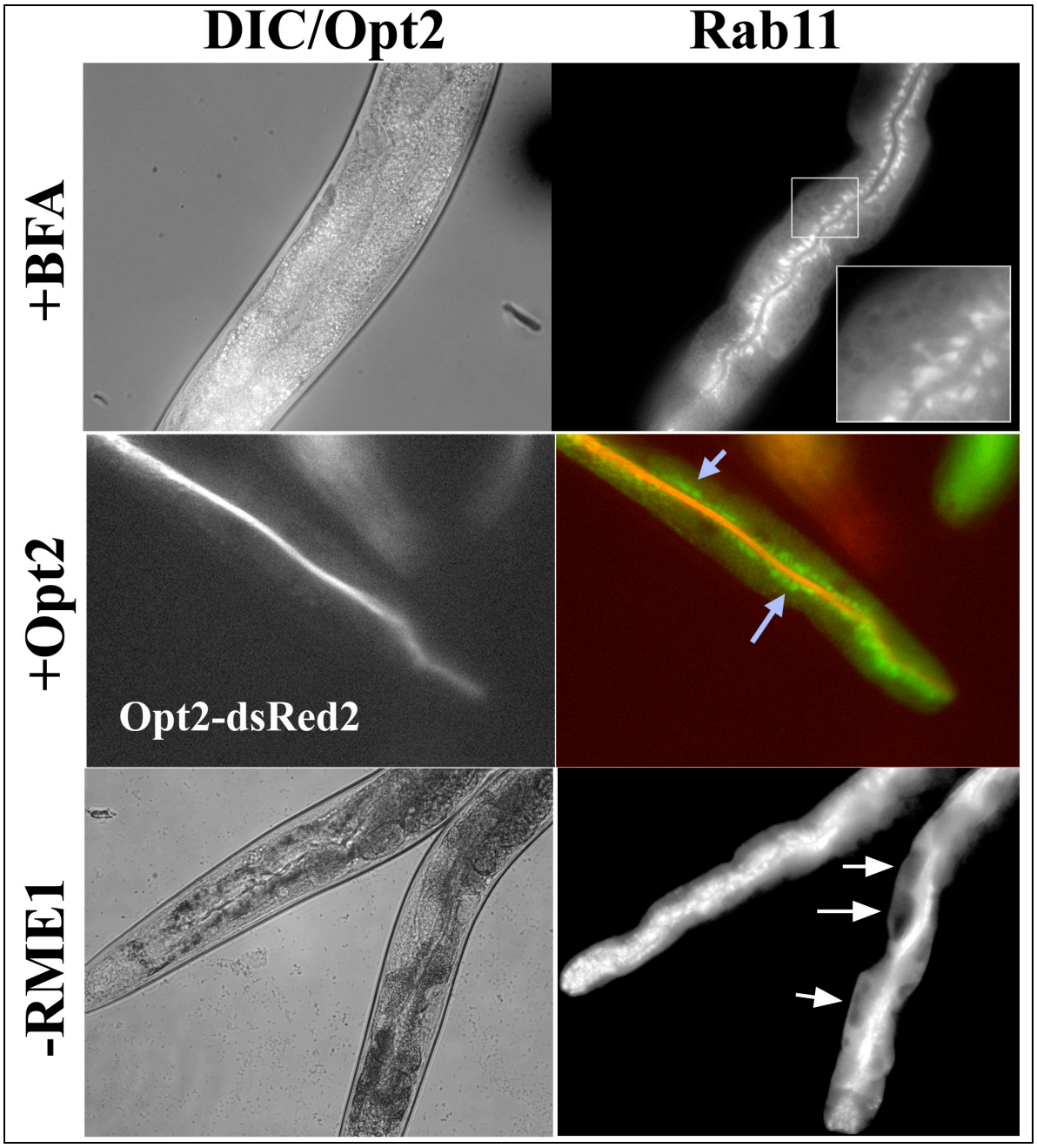


Figure 14
Rab11 positive endosomes change morphology upon addition of BFA or through the presence of a second marker (Opt2) but not after depletion of RME-1.

VI. I. 4. Small scale, proof-of-principle screen using the ARE marker Rab11

To be able to classify phenotypes which might result from the disruption of the normal function of the recycling endosomes, a small scale RNAi screen was devised. A list of target genes was assembled on the basis of (1) known or putative interaction with the small GTPase Rab11, (2) described recycling phenotypes in the literature or (3) genes functioning upstream of the recycling ensosome such as Rab5, for example. Predictably, not all candidate genes were conserved in C.elegans. Nevertheless, a total of 10 conserved target genes were chosen for the proof-of-principle screen (see table 4). YFP-Rab11 transgenic worms were soaked (see material and methods) in RNA specific for these genes and any changes in the morphology of the ARE were recorded using epiflourescence light microscopy. Worms were soaked over night and processed for imaging after 72h. Figure 15 summarizes the findings.

Selected Genes for knock-down in c.e. related to Rab11 compartment morphology			
Ce_GENE	Homo.S.ACCESS	Taget EXON	Description
Rbf-1	Nrip11	AAGTTCGTCG GATGGTGAAT…	Rabphilin-1 (splice 7a)
Y11D7A.14	Eferin	Atgtaatggagtgttggaaggaa…	Myosin class II heavy chain
R01E6.7(X)	FLJ10613	Tccctcgttaca…	cytoplasmic tail 1 to 226)
T04C9.1c	NP_741164	AAATCTTCCGGAACCCC..	PH, Src, RhoGAP domains
F07F6.4 (NP_495029)	ARFGAP3	GAGGAAGAAGATTTC….	Put. ARF GAP
C44H4.4	KIAA0701;	GTCAAACAACCCGGA	Homo.S: ICBP90 binding protein 1
F35H12.2a	GAPCenA	GCGATTGACCAACCT….	
Y17G7B.15b	Centaurin-beta2	ACACCTCCGCAAACA…	
C34D4.14	AAH34982.1	AGCACTATTCGACAC	E3-Ligase
Rab-5	Rab-5a	GTGTGCCTTGAC	Rab5

Table4
List of target genes which might affect Rab11 endosome localization or morphology.

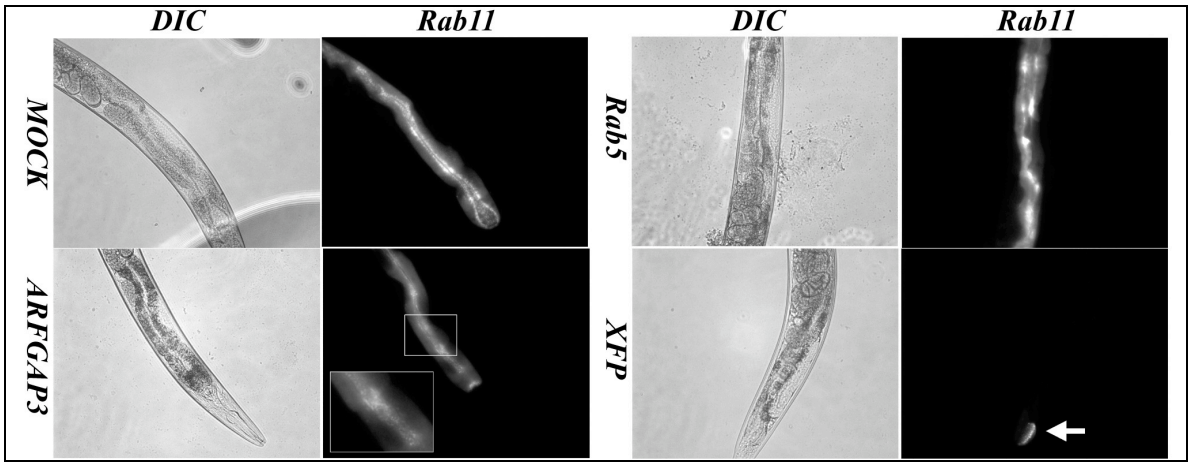


Figure 15
Phenotypes observable upon RNAi treatment using the Rab11 marker gene.
(1) Silencing of ArfGAP3: down-regulation of ARFGAP3 by RNAi resulted in a slightly decreased fluorescence intensity and a more diffuse pattern of subapical recycling endosomes. Loss-of-function of ArfGap3 could result in compromised uncoating of vesicles originating from the Golgi (REF). Since uncoating is a prerequisite to fusion, similar to a reduction in endocytosis, a smaller number of vesicles might be capable to fuse with the recycling endosome, resulting in the observed phenotype.
(2) Silencing of Rab5: the depletion of Rab5 resulted in a strongly clustered recycling endosome. The apical location was not compromised.
(3) XFP-RNA targets the expression of CFP, GFP and YFP-tagged markers serving as a positive control. After 72h virtually no worm remained fluorescent. Occasionally, a small patch of fluorescence 'survived' at the far posterior end of the intestine (arrow).

VI. I. 5. Small scale, proof-of-principle screen using the apical marker Opt2

A small proof-of-principle screen was designed to assess what phenotypes can be obtained using the secondary marker Opt2. A list of conserved genes functioning in various traffic steps were assembled (see table 5). Since exocytosis and endocytosis are intrinsically coupled, the genes were chosen to cover various functional groups in both pathways such as the exocyst complex, the retromer complex, as well as other genes known to be required for endo- and exocytosis (see table 5- 'remarks'). RNA producing bacteria from the Ahringer library were fed to nematodes which stably expressed the Opt2 marker gene. After 72h, worms exhibiting abnormal Opt-2 sorting were recorded. Figure 16 shows a range of phenotypes found in this small scale screen.

Nr.	*Gene*	*Access*	*Remarks*
1	GAP-2	ZK899.8a	Ras-GAP
2(*)	Fab1	VF11C1L.1	PI(3)5K is required for sorting of cargo into vacuole lumen
3	EEA1	T10G3.5	Early endosome antigen 1
4(F)	Rabaptin-5	K08B12.5	c-term region is well conserved. Rab5 binds there. N-terminus not conserved. Rab4 binds here.
5(*)	ceARF-6	Y116A8C.12	
6	Cup-5	R13A5.1a	CUP-5; an ortholog of the human mucolipin-1 gene, and is required for the normal, negative regulation of endocytotic uptake of markers from pseudocoelomic fluid.
7	CeCHC	T20G5.1	Clathrin heavy chain
8	CeAP2	F02E8.3	small chain of AP2
9	ceDynamin	C02C6.1	61% identical to human dynamin-I; expressed in motor neurons, intestine
10	CeRab5	F26H9.6	(From Ahringer library)
11	CeRab7	W03C9.3	Late endosome marker
12(*)	CeRab11	F53G12.1	Positive control
13	µ1B?	K11D2.3	Unc-101
14	CeKlp-3	T09A5.2	Molecular motor: minus ended
15	CeKlp-17	W02B12.7	Molecular motor
16	Rme-1	W06H8.1a	Basolateral recycling
17	Ce_auxilin	W07A8.3	CCV uncoating
18	CHE-14	F56H1.1	CHE-14; Apical transport
19	CeRab8	D1037.4	Rab-8; TGN-to-basolateral PM
20	CeRab21	T01B7.3	Rab-21; TGN-to-apical-PM
21(F)	PIK-1	T13C2.3	PI(4)K; PI(4)P required for exocytosis
22	Vps-34	B0025.1a	PI(3)K
23	ARFGAP3	F07F6.4	Putative ARF GAP
24	GAPCenA	F35H12.2a	Rab11-effector
25	NO Bacteria		Starvation Control
26	ARF1	B0336.2	Arf-1
27	Feeding Control	No insert	No insert control
28	Rrf-3	Control2	Feeding control
29	Sec-6		CeSec-6; exocyst complex
30	Vps-5	C05d9.1	Retromer complex
31	Sec-1	F27d9.1	
32	Sec-13	Y77e11a.13a	COP-II
33(F)	BIG1p	F11A10.4	Positive Phenotype in yeast screen
34	ARFGAP3	F07F6.4	ArfGap-3 (self-made clone)
35	CeRab5	F26H9.6	Rab5 (self-made clone)
36	XFP	GFP/CFP/YFP	Targets fluorescence

Table 5

List of feeding clones targeting genes which might affect Opt-2 sorting to the apical plasma membrane.

(*) marks clones that were contaminated in the original Ahringer library. An (F) represents clones where the correct identity could not be confirmed for other reasons.

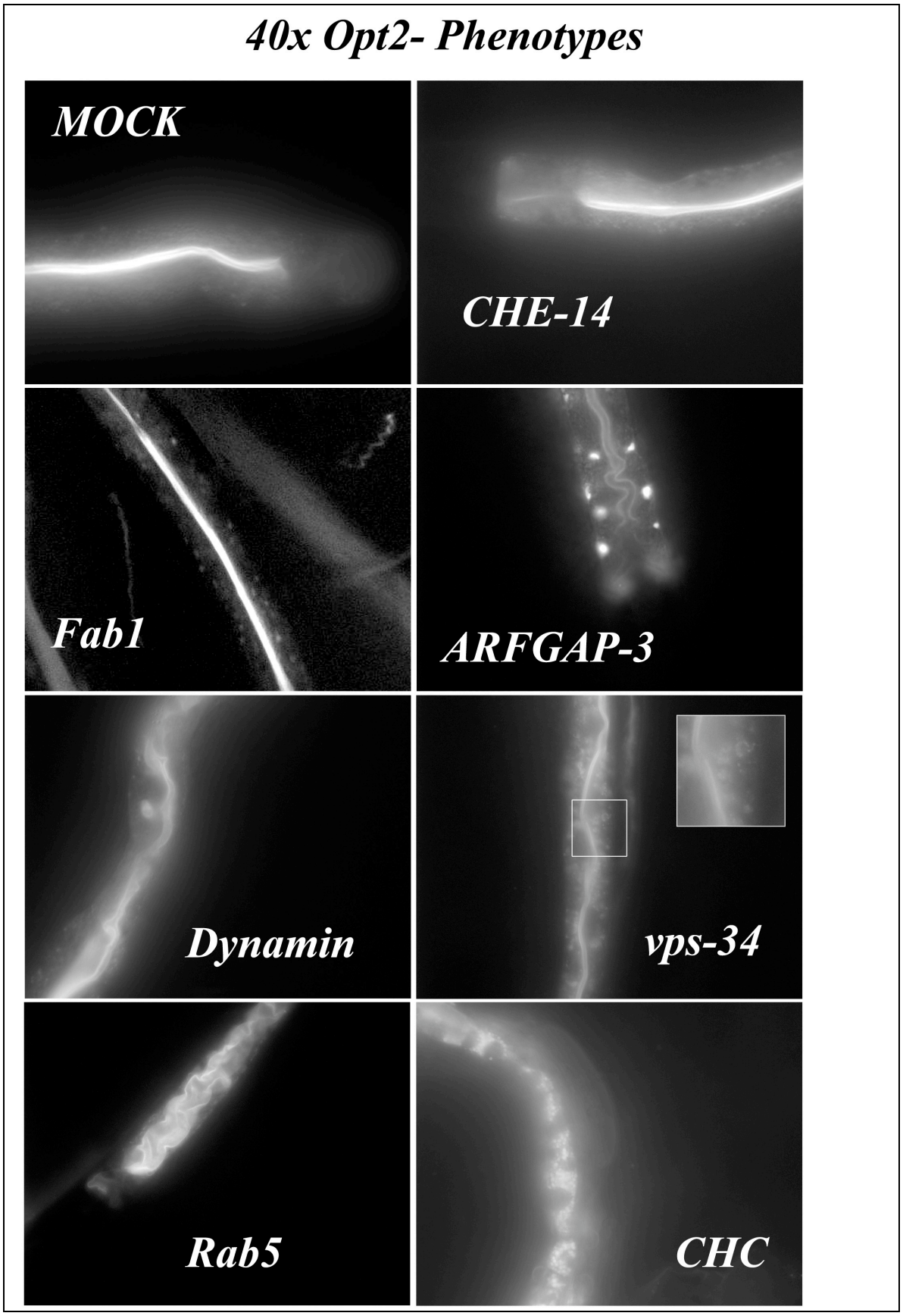

40x Opt2- Phenotypes
MOCK
CHE-14
Fab1
ARFGAP-3
Dynamin
vps-34
Rab5
CHC

Figure 16
Phenotypes observable upon RNAi treatment using the Opt-2 marker gene.
RNAi depletion of CHE-14 which has been implicated in apical sorting in epithelial cells results in a more diffuse Opt-2 localization. The diffuse staining could result from internal aggregates/vesicles. Fab-1 and ARFGAP-3 silencing results in distinct punctuate structures. Depletion of VPS-34 creates intracellular vesicles which contain a clear limiting membrane. Dynamin, Rab5 and CHC depletion causes a strong undulation of the apical plasma membrane of the intestine. Depletion of these essential genes will likely result in considerable cytotoxicity.

Several genes (most notably Rab8, Rab21 and Sec6) yielded no phenotype suggesting the presence of redundant molecules or pathways.

VI. II. Outlook C.elegans screening:

With the first phenotypes being found using the two markers Rab-11 under the Vit6 promoter and Opt-2 under its own promoter, the next steps will involve optimization for high-throughput screening. For this purpose, the Ahringer library has been amplified and re-arrayed from 384 to 96-well format with the aid of the TDS service at the MPI-CBG Dresden. Furthermore, a protocol has been established which allows fully automated handling of synchronized worms and RNAi feeding in liquid culture. At the time this manuscript was compiled, the first phenotypes were reproduced utilizing fully automated, large scale processing of worms and image acquisition based on an automated confocal microscope solution. Finally, a software solution was implemented which will allow high-speed image analysis of the images produced from the screen. Therefore, objective data will be harvested the quality of which can be assessed using statistical analysis.

VII. Concluding remarks

The work of this thesis focused on how the spacial localization of endocytic organelles is linked to their function in membrane traffic. Endosomes have a characteristic intracellular distribution and exhibit both short and long-range movements

with a wide range of velocities (Gasman et al., 2003; Nielsen et al., 1999; Valetti et al., 1999). The balance between plus- and minus-end directed microtubule motors determines the intracellular localization of early endosomes, recycling endosomes, late endosomes and lysosomes. KIF16B regulates the plus-end motility of early endosomes and their intracellular distribution. With the findings that alterations of KIF16B function also affected the transferrin cycle and transport from early to late endosomes, we provide evidence for a role of this motor in regulating endosome location and the balance between receptor recycling and degradation. Within polarized cells, recycling endosomes have a characteristic subapical position. Future experiments with RME1 mutants might clarify the structure and function of basolateral recycling endosomes. Surprisingly little is known what physiological significance relates to the asymmetric intracellular positioning of many organelles, such as the golgi, lysosomes and recycling endosomes, for example. Which molecules are expected to be identified in the C.elegans screen? One exciting possibility related to the first part of this thesis is that novel molecular motors may be identified which function in endosome localization and transport. Especially, since data from Eric Nielsen and coworkers predicted the existence of a minus end - directed kinesin specific for early endosomes (Nielsen et al., 1999). In addition, beyond the molecular motors, the hope is to obtain a comprehensive identification of candidate genes which function in polarized transport, endosome motility, maintenance of recycling endosome localization and morphology and provide a more complete insight into the recycling pathways in general.

VIII. Materials and Methods

VIII. I. Cell Biology and Biochemistry

VIII.I.1. Antibodies and other reagents.

Anti-tubulin antibody (1:250) was from Sigma-Aldrich, polyclonal anti-EEA1 (1:500) (Christoforidis et al., 1999a) and anti-LAMP1 (1:500) from PharMingen, anti-vps34 (Nielsen et al., 1999), sheep anti-EGF receptor (Fitzgerald; 1:1000) and rhodamine-conjugated transferrin from Molecular Probes. LY294002 and Wortmannin were obtained from Calbiochem-Novabiochem GmbH. Cell culture reagents were from Sigma-

Aldrich and Invitrogen. Other chemicals were from Sigma, unless specified.

VIII.I.2. Cell transfection, Immunofluorescence microscopy and time-lapse fluorescence video microscopy

HeLa cell transfection was carried out using Effectene (Quiagene)/DNA complex. Oligofectamine (Invitrogen) was utilized for esiRNA transfection. After 24 h (DNA) or 72 h (RNA), cells were used for analysis. For video-microscopy, cells transiently expressing GFP-Rab5 or YFP-KIF16B grown on glass coverslips, were transferred to custom-built aluminium microscope slide chambers just before observation. Time-lapse imaging was performed at 37°C and analyzed using customized software (Gasman et al., 2003).

VIII.I.3. cDNA cloning, sequence analysis, Northern blot and RNA interference

KIF16B was cloned from HeLa total mRNA using ATGAGCGATGGCATCGGTC and CTACCCCGTCCCGTGGCTG primers. The full-length construct was subcloned into pEYFP-N1 (CLONTECH, Palo Alto, CA) by ET-recombination. For the headless KIF16B constructs, carboxyl terminal sequences of human KIF16B were amplified from full-length KIF16B cDNA using PCR and subsequently cloned into pEYFP-N1 and pGEX-6P-1 to obtain KIF16B-ΔN (aa 396-1318) and KIF16B-ΔN2 (aa 219-1318) fusion constructs. The KIF16B-S109A construct was created by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The GST-KIF16B-PX construct was made by subcloning the c-terminus (aa 1169-1318) of KIF16B into EcoRI/SalI sites of pGEX-6P-1 following standard methods. All constructs were verified by DNA sequencing. Coils prediction of the protein sequences was run with weighing option window size of 28 for KHC, DdKIF1A and HsKIF16B and 21 for HsKIF1A. A Human tissue Northern blot purchased from BD Biosciences was probed for KIF16B mRNA (CTGGATTTGCGCCCAAAGAGCG, CAGAGAGAGGCGCTGGAGCGG) using the Dig Northern kit (Roche). KIF16B specific esiRNA was prepared as described (Yang et al., 2002) using GTAATACGACTCACTATAGGCAGGAGATCCTAGAG and GTAATACGACTCACTATAGGGCCCAGGGTGGAGTG primers.

VIII.I.4. Polyclonal antibody generation, affinity-purification, subcellular fractionation and immunoblotting

Truncated KIF16B-ΔN (aa 396-1318) protein was expressed and purified as GST-fusion protein in E.coli, cleaved from GST and injected into rabbits (Elevage Scientifique des Dombes, France). The antiserum was affinity purified using the antigen protein immobilized on Sulfolink beads (Pierce). An early endosomes enriched fraction was purified from HeLa cells mock-treated or treated with 100nM Wortmannin for 30min in 37C as described (Nielsen et al., 1999). The endosomes were subsequently analyzed using SDS-PAGE chromatography and immunoblotting or used for *in-vitro* motility studies.

VIII.I.5. In vitro motility-, microtubules gliding- and liposome assays:

The *in vitro* motility assay was essentially carried out as described (Nielsen et al., 1999) with the following modifications: KHMG (110 mM KCl, 50 mM HEPES-KOH pH 7.4, 2 mM $MgCl_2$, 10% Glycerol) was used as assay buffer. For preparation of the antifade solution, BRB80 buffer was substituted with KHMG and the solution was supplemented with 10% serum. The energy mix consisted of 75 mM creatine phosphate, 10 mM ATP, 10 mM GTP, 20 mM $MgCl_2$ in BRB80. Fluorescently-labelled, taxol-stabilized microtubules were perfused in a microscopy chamber to bind to the coverslip (Nielsen et al., 1999). Next, 10µl of 10% non-specific rabbit serum in antifade solution was perfused in the chamber followed by 10 µl of the assay mixture (2µl fluorescently-labeled endosomes (5mg/ml), 1µl energy mix, 6µl antifade solution and 1µl saturated haemoglobin solution in KHMG) and incubated for 5 min at RT. At least 3 movies per sample were recorded (60 frames, 2 sec intervals) and analyzed as described (Nielsen et al., 1999). When endosomal movement was analyzed using centrosome-grown microtubules, all 25 recorded motility events observed were plus-end directed. Liposomes were prepared as described (Klopfenstein et al., 2002) using DOPC, 2mol% PI(3)P (Echelon Research Laboratories) and 0.5mol% TRITC-DHPE (Molecular probes), diluted 1:100 in KHEM. To determine the directionality of the motor in the microtubule-gliding assay, HIS-tagged full-length KIF16B was purified using a baculovirus system

and 10µl of recombinant KIF16B (0.4 mg/ml) was left on a coverslip for 5 min, washed with KHMG and 3µl of the polarity-marked microtubules were added in BRB80 buffer containing 1mM ATP as described (Nielsen et al., 1999). To quantify the velocity of the motor-induced microtubule gliding, taxol-stabilized instead of polarity-marked microtubules were used.

VIII.I.6. Transferrin uptake, recycling and EGFR degradation

For the transferrin internalization assay, HeLa cells were transfected with KIF16B-EYFP expression vector or esiRNA specific for KIF16B or a pBluescript vector sequence (mock). At 16h (DNA) or 74h (esiRNA) post-transfection, cells were starved for 4 h in CO2-independent DMEM medium containing 0.2%BSA and then incubated with Alexa568-labeled Tfn (Sigma-Aldrich) at 0.5 mg/ml for the indicated times. For biochemical uptake analysis, starved cells were allowed to internalize biotinylated Tfn (10µg/ml) for the times indicated. Cells were placed on ice, washed, lysed and the total cell extract was incubated with affinity-purified, Ruthenium-labelled, sheep-anti-human Tfn antibodies (SAPU, Scottland) and subsequently analysed using an ECL-Analyser System from Igen Inc. (Rockville, MD). The amount of internalized Tfn was standardized with respect to total protein concentration of the lysate and expressed as percent of the amount of internalized Tfn in control cells at t=40min (n=4; mean +/- SD from two independent experiments). The Tfn recycling assay was carried out as described (de Renzis et al., 2002), except that cells were rapidly washed once at 37°C with CO2-indep. DMEM with 100µg/ml human holo-Tfn (n=4; mean +/- SD from two independent experiments). EGF stimulated EGFR uptake and degradation was either studied by confocal microscopy and image processing (Bache et al., 2003) or measured biochemically. For EGFR degradation, ($5x10^5$, 5cm dishes) HeLa cells were transfected for 24h with 400ng of the indicated plasmid, using Effectene(R). The cells were then incubated with or without 100ng/ml EGF for 30 min, washed and chased for 6h in normal growth medium containing 10µg/ml cycloheximide. Cell extracts were then analysed by SDS-PAGE and Western blotting with anti-EGFR antibodies.

VIII.II. C.elegans methods

Methods not explicated listed here are standard methods found in the book:
C. Elegans II : Monograph 33 (Cold Spring Harbor Monograph Series,33) by Donald L. Riddle et al.
or are posted online: http://elegans.swmed.edu/

VIII.II. 1. Strain generation

The C.elegans strain Unc-119(ed3) was received from the Caenorhabditis Genetics Center. The plasmids listed in table 3 were generated following common cloning protocols of molecular biology. Rather than listing here the several hundred PCR primers utilized to generate the expression vectors, the interested reader may contact me directly for a digital vector map and cDNA of the various plasmids (hoepfner@mpi-cbg.de).

Plasmids carrying the different markers fused to CFP, GFP, YFP or dsRED2 were bombarded following standard methods (Praitis et al. Genetics 2001) into the Unc-119 strain to obtain low-copy integrated transgenic lines. Following the biolistic transformation, strains were crossed to generate a line carrying both markers (Rab11 and Opt-2) in the same worm.

VIII.II. 2. Synchronization of worms

This follows general protocols for liquid culture and synchronization. Briefly, transgenic worms were transferred from plate into S-Basal/C600 liquid broth medium. After reaching sufficient density, the worms were extracted with a 40% sucrose solution, washed several times in M9 medium and subjected to bleaching. The isolated embryos were allowed to mature to the L1 stage in the absence of food and finally used for starting a synchronized liquid culture.

VIII.II. 3. Plate Feeding method

The transgenic worm line was maintained according to standard protocols (Brenner, s. Genetics 77, 1974). For the proof of principle, 33 genes involved in membrane trafficking were knocked-down using the standard feeding protocol (Kamath et al, 2000, genome biology). Briefly, bacteria were grown in LB medium plus 50ug/ml ampicillin over night and subsequently plated on NGM agar plates including 25ug/ml Carbenicillin and 1mM IPTG. Finally, transgenic worms obtained from a synchronized liquid culture were transferred to the plates. The plates were incubated at 20C until F0 and F1 hermaphrodites could be imaged using a Zeiss inverted epiflourescence microscope.

VIII.II. 4. RNA soaking method

L4- Worms were soaked in 0.2M sucrose and 0.1% phosphate-buffered saline, 10ug of siRNA and 5% DOTAP. After 12h at 16C the worms were recovered on plate containing OP50 E.coli, grown at 20C and the adult F1 generation was analyzed.

VIII.II. 5. Automated liquid feeding method

The Ahringer RNAi feeding library (Kamath and Ahringer, 2003) was bought from MRC (http://www.hgmp.mrc.ac.uk). The 16,757 clones contained in this library correspond to roughly 86% of the *C. elegans* genome. The original library was re-arrayed with the help of the TDS-Dresden from 384-well into 96-well format.

Bacterial strains harboring the individual knock-down plasmids were cultured in 96-well plates and induced with 0.5mM ITPG for 4h at 37C. The bacteria were spun down and washed twice with NGM medium. Next, worms from a frozen, synchronous (L1 stage) liquid culture were dispensed into each well and allowed to reach adult state at 16C. For image acquisition, the worms were washed four times using a Tecan robotics platform, paralyzed using Levamisole and imaged using an automated inverted confocal fluorescence microscope – the OPERA station.

IX. Publication record of thesis research

Part of this thesis has been published:

Hoepfner, S., Severin, F., Cabezas, A., Habermann, B., Runge, A., Gillooly, D., Stenmark, H., and Zerial, M. (2005). Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B. Cell *121*, 437-450.

X. Abbreviations

AP-1: Adaptor Protein-1
Arf: ADP- Ribosylation Factor
BFA: Brefeldin A
CCV: Clathrin Coated Vesicles
COP: Coatomer coat protein
DMEM: Dulbecco's modified Eagle's medium
DMSO: Dimethylsulfoxide
DTT: Dithiothreitol
ECV: Endosomal carrier vesicle
EDTA: Ethylenedioxy-diethylene-dinitrilo-tetraacetic acid
EGF: Epidermal growth factor
Eps15: Epidermal growth factor protein substrate 15
ER: Endoplasmic Reticulum
FCS: Foetal calf serum
FRET: Fluorescence resonance energy transfer
FYVE: Fab1p, YOTB, Vac1p, and EEA1
GAP: GTPase activating protein
GDI: GDP-dissociation inhibitor
GDP: Guanine-nucleotide-diphosphate
GEF: Guanine nucleotide Exchange factor
GFP: Green Fluorescent Protein
GGA: Golgi-localized, γ-ear-containing, Arf-binding
GTP: Guanine-nucleotide-triphosphate
HEPES: N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
Hrs: Hepatocyte growth factor regulated substrate
LDL: Low Density Lipoprotein
MTOC: Microtubule organizing centre
MVB: Multivesicular body
NSF: N-ethylmaleimide-sensitive factor
PAGE: Polyacrylamide gel electrophoresis
PBS: Phosphate-Buffered Saline
PCR: Polymerase chain reaction
PDGF: Platelet-derived growth factor
PIK: Phosphatidyl-Inositol Kinase
PtdIns: Phosphatidyl-Inositol
PtdInsP: Phosphatidyl-Inositol Phosphate
Rab: Rat Brain
SDS: Sodium dodecyl sulfate

SNARE: Soluble NSF-attachment factor receptor
SNX: Sorting Nexin
TF: Transferrin
TGN: Trans-Golgi Network

XI. References

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